MEMOIRS
OF THE
FACULTY OF FISHERIES
HOKKAIDO UNIVERSITY

Vol. 3, No. 2

THE FACULTY OF FISHERIES, HOKKAIDO UNIVERSITY
HAKODATE, JAPAN
1955

MEMOIRS
OF THE
FACULTY OF FISHERIES

Vol. 3, No. 2

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STUDIES ON THE MANUFACTURE OF ALGIN FROM BROWN ALGAE

NOBORU SUZUKI

Faculty of Fisheries, Hokkaido University, Japan

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INTRODUCTION

Algin was first extracted by STANFORD[1)] from the bladder wrack (*Fucus vesiculosus*) with sodium carbonate. His process may be described as follows: drift-weed is macerated, with or without heating, for twenty-four hours with one-tenth of its weight of sodium carbonate. At the end of this period, the plants are completely disintegrated and a very viscous, semi-gelatinous mass is obtained. The alginic acid is precipitated from the filtrate by addition of either sulfuric or hydrochloric acid.

There are available only a few theoretical studies regarding the process for

manufacturing algin, although some improvements of manufacturing method have been reported.

One of the simpler ways for obtaining algin is by pretreating algae first with dilute hydrochloric acid in order to remove any soluble mineral salts, mannit and impurities ; then the algin is extracted by using a solution of sodium carbonate resulting in a viscous extract ; the extract is filtered, and finally the algin is precipitated by treating it with hydrochloric acid.

DILLON[2] has described another easy method of preparation as follows ; the weed is first extracted for short time with boiling water in order to remove fucoidin. It is then soaked in dilute hydrochloric acid for a day, after which the alginic acid is removed with ammonia. Wet weed was commonly used for these extractions, but GLOESS[3] considered that the maximum extraction was achieved when the weed was dry rather than wet. Fresh wet weed has been used preferably by the factories in Europe and America in order to obviate the drying cost which may outweigh the advantage of using dried weed, whilst dried algae have been used in Japan to manufacture algin as they are produced abundantly for preservation as food.

There have been reported several procedures of pretreating algae as mentioned below. These pretreatments have been worked out without any consideration as for the mechanism of algin manufacture, only for the purpose of extracting mineral matter and pigment or other impurities and of making maceration with sodium carbonate easy. Pretreatments have been reported with lime water and then with water, with acid and hydrocarbon,[4] with hydrogen peroxide or chlorine in neutral or acidic solution,[5] with sea water containing aluminium sulfate in weak acid[6] or with saturated solution of sodium bisulfate for 2–10 hours at 80–90°C.[7]

As for the details of commercial process in Europe and Japan, no published report is available ; on the other hand, a report concerning the commercially used processes in America was published by CHAPMAN.[8] He described two processes, of which one was so-called "cold process" and the other was "Le Gloahec-Heyter process." His description is summarized here briefly as follows.

In the first process, or cold process, fresh kelp is first leached for several hours with 0.33 per cent hydrochloric acid. After being chopped and shredded, the leached kelp is digested with soda ash solution (40–50 lb per ton of fresh kelp) at a pH of about 10 ; this first digestion occupies about 30 minutes and it is then repeated. The crude pulp is shredded again and six volumes of treated water are added at pH of 9.6–11. At this point the crude fibrous material can be dried and sold as crude sodium alginate.

To obtain a purer product the liquor is filtered, using filter aid and presses, and the temperature may be raised to 120°F to assist the process. The filtered liquor is added to a 10–11 per cent calcium chloride solution under constant agitation, and when the agitation is stopped calcium alginate slowly rises to the top. The remaining liquor is drained off and more water and bleacher (sodium hypochlorite) is added to the

precipitate. The bleached precipitate is separated off and added to a 5 per cent hydrochloric acid solution (1 part alginate solution to 42 parts acid). This converts the calcium alginate into fibrous alginic acid, which is purified by the use of dilute acid between each screening. The alginic acid so produced can be filtered and stored or else converted into salt by treatment with carbonate, oxide or hydroxide.

In the Le Gloahec-Heyter process the raw kelp can be either fresh or dried. To one part of kelp three parts of 0.8–1 per cent calcium chloride solution are added, the solution being either hot or cold. The function of the calcium chloride solution is to remove laminarin, mannit and other salts. These salts and the calcium are then removed by washing with softened water, after which the remaining material may receive an additional treatment with 5 per cent hydrochoric acid in order to dissolve any residual alkaline earth salts. It is washed once more with softened water, and is then digested with 4 per cent soda ash solution in the proportions of two volumes solution to one volume of kelp. Lixiviation is continued for about two hours at 104°F and the kelp is macerated at the same time until it is reduced to a paste.

The resulting paste is diluted with water in the ratio 3 : 7, and after being beaten into a homogenous suspension it is vigorously aerated, but if ozone or hydrogen peroxide is used it is merely agitated mechanically. The liquor is then passed continuously at high speed through a centrifuge, where it is charged with air bubbles, and then led into a clarifying tank. Here the cellulose particles agglomerate to form a floating cake and the liquor is removed.

The coloured liquor is decolourised by addition of an absorbent jelly, usually made of hydrated alumina, gelatinous silica and aluminium alginate. The jelly is removed by centrifuging and can be reclaimed by various methods.

The alginic acid is now precipitated by running it into mixing baffle where it meets a stream of strong hydrochloric acid, arranged in such a way that the precipitate runs at once into another tank. All through this process the pH of the solution is maintained at 2.8–3.2. The precipitated alginic acid is placed in baskets and drained, after which it is purified and dried.

TAKAHASHI[9] described pretreatment of brown algae as follows. Raw weeds should be washed with water in order to remove dirt on the weeds, and the soluble materials of the weeds, such as iodine, mannit, potassium chloride, sodium chloride, laminarin or fucoidin were extracted from the weeds by this treatment. Extractive power would be enhanced by the addition of some chemicals in pretreating water; hydrochloric acid was the most excellent one.

Pretreating of algae with certain solution should be done to prevent the plant material from swelling in order to enhance the efficiency for extraction of soluble mineral salts, mannit or impurities.

Algin has been manufactured by macerating algae with alkaline solution, such as sodium carbonate, sodinm hydroxide or ammonium hydroxide solution. TAKAHASHI[10]

attempted to digest algae with neutral salt, ammonium oxalate, as follows. He attempted to extract algin with oxalate in the same way as in the case of extracting pectic constituents[11] from higher plants because alginic acid fairly resembles pectic acid in respect to chemical configuration.

It was explained that insoluble alginate, such as calcium or aluminium alginate, in brown algae was dissolved into soluble alginate by the following double decomposition (1):

$$\text{Ca-Alginate} + \text{M-Oxalate} = \text{Ca-Oxalate} + \text{M-Alginate} \quad \cdots\cdots \quad (1)$$

where, "M" represents alkali metals or ammonium radical. He did not carry out any further theoretical investigation.

It is usual to pretreat algae with water, hydrochloric acid or other solutions in order to extract potassium salts, iodine, mannit and other impurities and to treat them with sodium carbonate to make them easily macerable, but little consideration has been given to the matter of converting algin into a certain definite form. In previous paper,[12] the present worker reported on the significance of converting algin in brown algae into certain definite forms e.g., free alginic acid or calcium alginate, by pretreating algae with some solution.

In the present paper are reported the process of manufacture of algin from brown algae and the theory thereof under the condition of converting algin in brown algae into free alginic acid and calcium alginate by pretreating the plants with hydrochloric acid or calcium chloride solution. The manufacturing conditions and by-products in algin industry are also reported.

ACKNOWLEDGEMENT

The author wishes to express his heartiest thanks to Professor Yukihiko NAKAMURA and Professor Yataro OBATA for invaluable advices and constant encouragement throughout the course of the present study. The author's cordial thanks are due to Professor Jun TOKIDA for reading over the manuscript. The author is much indebted to Messrs. Miki OGURO and Shohei TAMURA for their assistance in carrying out the experiments; to the late Professor Chiyoichi KANDA for the identification of the algal materials; and to Miss Michiko KONISHI for drawing the figures.

I. CHEMICAL STUDIES ON RAW MATERIALS OF ALGIN MANUFACTURE

1. Occurrence of Algin in Brown Algae

The term algin was first applied by STANFORD[1] to a mucilageous polyacid occurring in *Laminaria* fronds which was soluble in dilute soda solution and precipitated by mineral acids. KYLIN[13] applied the term to a supposed insoluble calcium salt which he assumed to occur in the cell wall; he stated that it was insoluble or very slightly soluble

in water and suggested that hot water would extract from the weed an alkali salt of alginic acid produced from calcium salt, but gave no experimental evidence. Moreover, he explained that soluble sodium alginate was produced by double decomposition between insoluble calcium alginate and sodium carbonate. BIRD & HAAS[14] stated that hot water did not remove all the alginic acid from the cell wall, even though they could obtain 2 g of an algin-like substance from 180 g of *Laminaria* fronds. Analysis of this substance showed that it contained 3.46 per cent of Ca and 0.45 per cent of Mg, together with undetermined quantities of alkali metals. From these facts they stated that the above-mentioned substance was to be considered as a water soluble calcium, magnesium, sodium and potassium salt of alginic acid, since it yielded on acidification a gelatinous precipitate resembling alginic acid in every respect.

BIRD & HAAS concluded that the cell wall of *Laminaria* contained alginic acid in two forms, one in the form of soluble calcium, magnesium, alkali metal salt being present only in such an amount as about 1 per cent, and the other in the free state being met with in the material extracted by cold diluted sodium carbonate.

The state of the algin in brown algae can not be decided from the above mentioned facts, because both alginic acid and insoluble alginates undergo cation exchange partially or perfectly with electrolytes as mentioned later.

MIWA[15] investigated the calcium and magnesium contents of six species of brown algae, and found in all of the species but one that such contents did not exist in sufficient quantities to combine with alginic acid in the fronds. He suggested that some amounts of algin existed in some other states of combination than calcium or magnesium alginate, whilst he detected calcium in the cell wall of the fronds through a microchemical examination.

Figs. 1 and 2 show the results of the author's microchemical test for the presence of calcium in the cell wall of *Laminaria angustata* KJELLM. Crystals of calcium oxalate were found in the intercellular spaces in the sections that had been treated with 0.5 per cent oxalic acid.

Since algin is considered to exist as an intercellular substance in the cell wall of brown algae, the above-mentioned examination suggests the existence of calcium alginate in the cell wall.

The existence of algin in the cell wall of *Laminaria angustata* was detected by the following two methods: 1. Frozen microtome sections of the fronds were put into 1 per cent H_2SO_4 solution and then into 2 cc of I_2KI solution (1 g of iodine was dissolved in 100 cc of 5 per cent KI solution) and the blue colouration of the wall was examined under the microscope. A preliminary examination[16] has proved that the algin, especially calcium alginate, was coloured blue by the above reagents. Fig. 3 shows the result of this test which suggests the existence of calcium alginate in the cell wall of *L. angustata*. 2. Frozen microtome sections were steeped in 0.5 per cent calcium chloride solution and boiled for 15 minutes, whereupon almost all of the algin was

converted into calcium alginate. Thus treated sections were microchemically examined by the above described method. The results are shown in Fig. 4. The blue colouration of the walls is more marked than that shown in Fig. 3.



Fig. 1

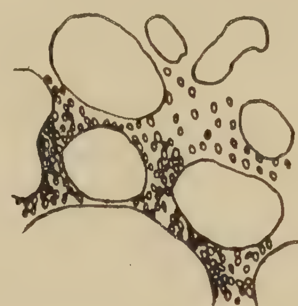


Fig. 2

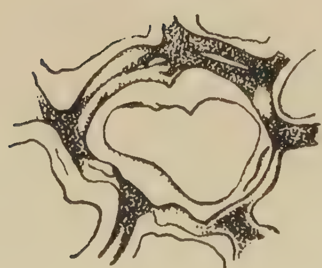


Fig. 3



Fig. 4

Fig. 1. Calcium oxalate crystals in the intercellular spaces of the parenchymatous tissue

Fig. 2. Calcium oxalate crystals in the intercellular spaces of the filamentous tissue

Fig. 3. Ca-alginate in the walls of parenchymatous cell is detected by blue colouration (dotted points) due to $I_2KI-H_2SO_4$

Fig. 4. Ca-alginate in the walls of parenchymatous cells previously treated with 0.5% $CaCl_2$ solution (dotted points) due to $I_2KI-H_2SO_4$

It may therefore be said that the algin occurs partly or mostly as calcium alginate in the cell wall of *L. angustata*, and that the algin which would be converted into calcium alginate through the treatment with calcium chloride solution is also apparently present there. The algin may be combined to form complex compounds with mannit, proteins or amino acids and other components of algae. It is then considered that the algin occurs in not so simple state but very complicated state, and that the

mechanisms of algin manufacture can not be studied without first causing the algin in algae to convert into certain definite states of combination.

2. Chemical Components of Brown Algae

Chemical analyses of brown algae have been repeatedly reported by many authors, but there are only a few works in which the preparation of materials has been made under well-thought-out management and the seasonal variation of the components has been reported. Even the specific differences could not be discussed without considering the season or locality of collection, because the seasonal and local differences of components of brown algae are fairly remarkable.

The present author analysed a number of brown algae especially those which are commonly used as the raw materials of algin manufacture and he studied the seasonal, local and specific differences of the components.

(i) *Experimental*

Preparation of samples:

The brown algae for the present study were collected by the author at various localities along the coast of southern Hokkaido and identified by the late Professor Chiyoichi KANDA in 1943. Collected algae were washed with sea water and dried on rocks or straw mats. Dried materials were shattered and analysed.

Analytical methods:

Moisture and ash contents were analysed by ordinary methods.

Potassium contents were determined by the author's own method[17] as follows: The ash obtained from 10 g of the material was extracted by hot water and the extract was filled up to 200 cc with water. Ten cc of 1 n tartaric acid solution and 5 cc of n/2 sodium acetate solution were added to 10 cc of the extract which had been pipetted into a conical beaker. After mixing the solution, 12 cc of 95 per cent ethanol was added in the course of 3 minutes, with gentle shaking. Produced $KH(CHOHCOO)_2$ crystals were collected on a glass filter under reduced pressure and washed several times with 10–15 cc of 67 per cent ethanol. The separated crystals were dissolved in hot water and titrated with 0.1 n NaOH solution. Potassium content was calculated as K_2O by the following equation.

$$K_2O\ \% = a \times 0.47/m$$

where, a is volume (cc) of titrated 0.1 n NaOH solution and m is weight (g) of the sample.

Iodine contents were determined by the author's method[18] as follows: Each 5 cc of 10 per cent H_2SO_4 and 0.5 per cent $NaNO_2$ solution was added to 25 cc of sample solution, which contained 1–10 mg iodine, in an Erlenmeyer flask with stopper and the mixture was allowed to stand for 5 minutes. Ten cc of 10 per cent urea solution was then added, the flask was stoppered and the solution was shaken gently to mix well and

was allowed to stand for 30 minutes. After that time, the NO_2 reaction with Griess reagent has become negative. Liberated iodine was determined through iodometry with 0.01 n $Na_2S_2O_3$ solution. Principle of above mentioned process is based on the following reaction, where excess of NO_2^- is decomposed by urea.

$$(NH_2)_2CO+2HNO_2=2N_2+CO_2+3H_2O$$

Chlorine contents were determined by titration with silver nitrate solution as ordinarily, and total halogen contents were calculated as chlorine.

Crude fat and crude protein contents were also determined by ordinary methods.

Mannit contents were determined by the author's improvement of Smit's method[19] to prevent interference by ferric ion. The method was as follows: Each 25 cc of 4 n NaOH solution and 12.5 per cent $CuSO_4$ solution was added to 50 cc of sample solution and the mixture was shaken for several minutes, and allowed to stand for 15 hours. After that period, 5 cc of 6 n CH_3COOH and 5 cc of 5 per cent KI solution were added to 25 cc of the filtrate. The liberated iodine was determined with 0.01 n $Na_2S_2O_3$ solution, and the amount of mannit was calculated from Smit's table as usual.

Alginic acid was determined as follows: Ten grams of the materials were macerated with 3 g of sodium carbonate anhydride and 250 cc of water on the boiling water bath for 2-5 hours until the weeds were completely disintegrated. Dilute hydrochloric acid solution, (1 : 2), was added to the filltrate after diluting the disintegrated mass with water to 600–700 cc until the alginic acid gel was completely precipitated. The precipitated gel was washed with hot water several times, with 95 per cent ethanol, and weighed after drying to constant weight at 95°C.

(ii) *Results and discussion*

Analytical results are shown in Table 1. From the results the following discussion is suggested.

It is shown that a high content of ash, especially potassium salts, and the existence of alginic acid, mannit and iodine are characteristic of brown algae.

Potassium contents of Laminariaceae are generally higher than that of Fucaceae. Since the seasonal variation of iodine contents is very remarkable as is described later, the difference in iodine contents among various species can not be here discussed. However, iodine contents are characteristically high in Laminariaceae, amounting to 0.1 per cent in their maximum season.

Since the seasonal variations of crude fat, curde protein and mannit are remarkable as is later described, the difference in their contents among various species can not be here discussed, although the contents of both fat and protein may here be stated to be poor in their maximum period in spring. Mannit contents in brown algae amount to 20–30 per cent in the maximum, while they amount to only several per cent in their minimum season as is described later.

Alginic acid contents in brown algae do not show obvious seasonal variation as is

described later. Alginic acid contents in Laminariaceae are generally larger than those in Fucaceae, that is to say, alginic acid contents amount to about 20–30 per cent in the former and about 15–20 per cent in the latter.

3. Seasonal Variation of Chemical Components

The seasonal variations of the chemical components of *Laminaria japonica, L. angustata, Alaria crassifolia* and *Costaria costata* were investigated. The materials were collected at a definite station in Muroran Bay.

Results and discussion

The results of the investigation are shown in Figs. 5–11.

Seasonal variations of laminarin and mannit contents of *Eisenia bicyclis* were reported by NISHIZAWA.[20–22] He stated that mannit and laminarin of *Eisenia bicyclis* were considered not to be direct photosynthetic products but reserve substances. From this viewpoint, he investigated the variations of mannit and laminarin contents in relation with season and growth stage. LUNDE[23] reported on the seasonal variations of the contents of ash, laminarin, mannit and alginic acid of *Laminaria digitata*. His obtained results are summarized as follows. Laminarin contented to 20 per cent in autumn, but it decreased to almost nothing in spring; mannit content amounted to 20

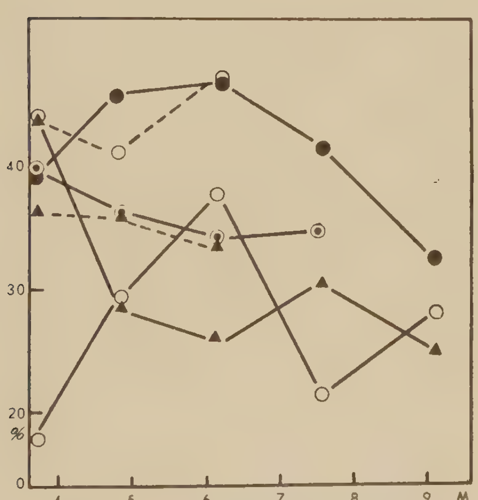


Fig. 5. Seasonal variation of ash content

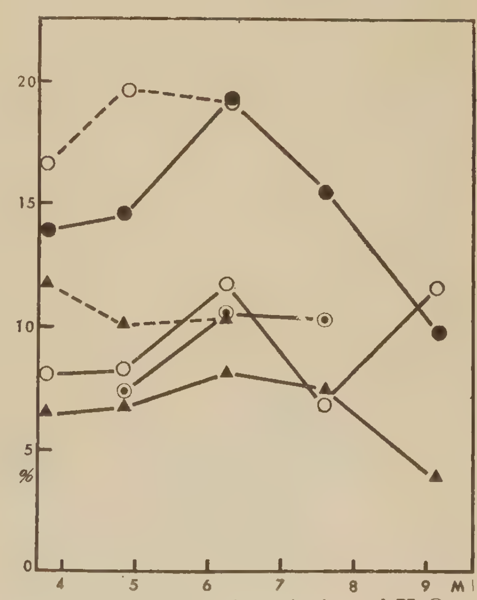


Fig. 6. Seasonal variation of K_2O content

●-------- *Laminaria japonica* ARESCH. ○-------- *Laminaria angustata* KJELLM

▲-------- *Alaria crassifolia* KJELLM. ⊙-------- *Costaria costata* (TURN.) SAUND.

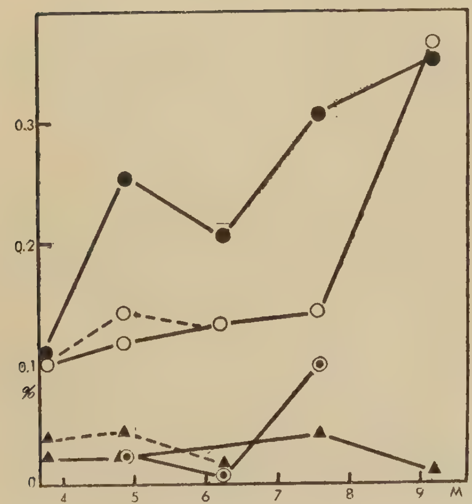


Fig. 7. Seasonal variation of iodine content

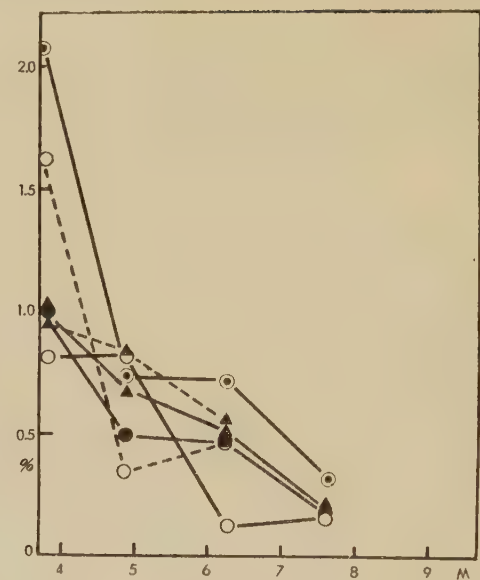


Fig. 8. Seasonal variation of fat content

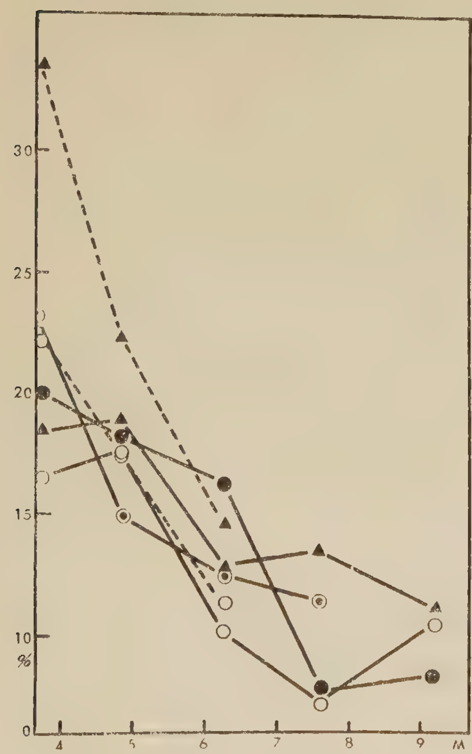


Fig. 9. Seasonal variation of protein content

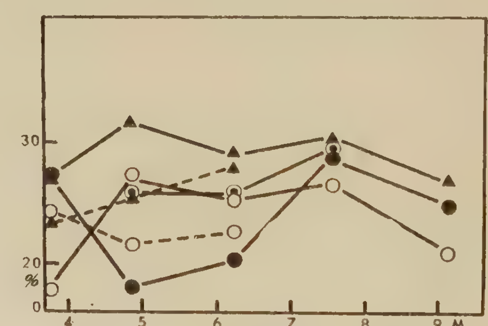


Fig. 10. Seasonal variation of alginic acid content

●------ *Laminaria japonica* ARESCH.
▲------ *Alaria crassifolia* KJELLM.
○------ *Laminaria angustata* KJELLM.
⊙------ *Costaria costata* (TURN.) SAUND.

per cent in summer and it decreased to 4–6 per cent in winter. The ash content showed maximum in spring and minimum in autumn. Alginic acid content attained to over 30 per cent in spring and decreased to under 20 per cent in autumn.

From the author's results, the following points are noteworthy. Because the seasonal variations of the chemical components interact upon each other regarding their contents, the absolute amount of each component in the materials can not be discussed but their relative contents can be. Very obvious seasonal variations are observed in the amounts of iodine, mannit, protein and fat. The former two components show minimum amounts in spring and evidently increase toward autumn. On the contrary, the latter two show their maxima in spring and minima in autumn. The above mentioned relation is considered to be significant in the life of brown algae, because the photosynthesis of plants is at its maximum in summer and the metabolic processes are at their minima in winter. The contents of ash, potassium and alginic acid vary seasonally to some degree, but the variations can not be distinguished from the variations which may be caused by alternative relations among other obviously varying components.

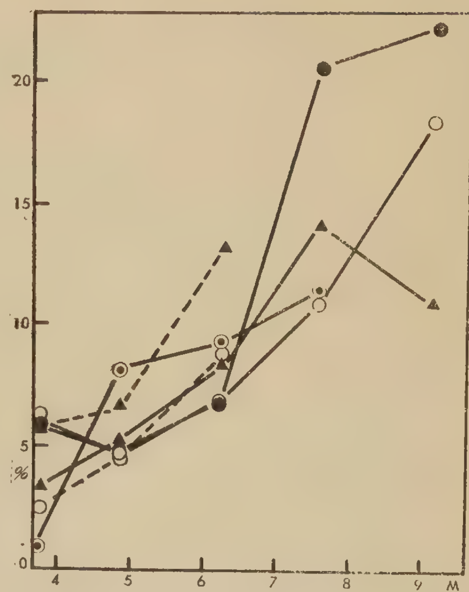


Fig. 11. Seasonal variation of mannit content

●------ *Laminaria japonica* ARESCH.
○------ *Laminaria angustata* KJELLM.
▲------ *Alaria crassifolia* KJELLM.
⊙------ *Costaria costata* (TURN.) SAUND.

Alginic acid, from the above noted results, would be considered a standing component of brown algae because its content does not alter very obviously with season or species. Alginic acid is considered to exist as an intercellular substance in the cell wall of brown algae, and so to play some important role in thier skeleton. As for the role of algin in brown algae, further studies should be made.

II. MECHANISM OF ALGIN MANUFACTURE

Because algin in brown algae exists in various types of combination as described above, it is desirable to convert algin in brown algae into a certain definite form in order to study the mechanism of algin manufacture as well as manufacture algin in practice. It is practically a useful method to pretreat algae with hydrochloric acid or

calcium chloride solution. Theoretical and practical studies on algin manufacture reported in this paper were carried out by pretreating the material with the above-mentioned reagent solutions and converting the algin in brown algae into free alginic acid and calcium alginate.

1. Dissolution of Free Alginic Acid

It is customary to pretreat algae with 0.1 n hydrochloric acid solution, but that is done as a general practical step without any consideration of converting various types of algin in brown algae into free alginic acid. The pretreatment has usually been made to extract potassium salts, iodine, mannit and pigment or other impurities, but the author proposes to pretreat algae with some certain solution in order to convert algin into a definite type of combination. Having this point in mind, fundamental investigations on the pretreatment of algae with hydrochloric acid were carried out with purified alginic acid.

(i) *Preparation of alginic acid*

Alginic acid was prepared by the author's own method[24] as described later, and it was purified well through repetition of dissolution by NaOH solution and of precipitation by HCl solution following the ordinary process, and then dialysed in distilled water till Cl ion reaction was eliminated.

(ii) *Dissociatton of saturated alginic acid solution*

The dissociation degree of alginic acid as determined for the first time by PAULI & STENRBACH[25] was 1.69～0.270 in the concentrations of alginic acid $59.7 \sim 1.6 \times 10^{-3}$ mol. SEIYAMA[26] determined the pK values and dissociation degrees of alginic acid solutions, as 2.87～4.60 and 0.285～0.316 respectively in the concentrations of alginic acid $11.88 \sim 0.19 \times 10^{-3}$ mol. He calculated the above mentioned values from the values of pH and from electric conductivities; the values obtained by both methods agreed fairly well with each other. The author determined the dissociation degree, pH, ionization constant and pK of the saturated solution of alginic acid.[27]

The concentration of the solution was determined through the titration curves with 0.01 n NaOH solution. The dissociation degrees were calculated from the values of hydrogen ion concentration obtained from pH values and the concentration of alginic acid. The pH values were measured by glass electrode pH meter made by MITAMURA Shoten. The ionization constants were calculated from pH values granting alginic acid to consist of mono-mono-valent mannuronic acid, and then the values of pK were obtained from ionization constants.

The above mentioned values obtained from saturated alginic acid solutions are shown in Table 2. Each solution is prepared by saturating 0.5 g of alginic acid in 100 cc of redistilled water, "A" represents the solution allowed to stand for 20 hours at 25°C after boiling for 30 minutes, "B" represents the solution allowed to stand for an hour at 25°C after boiling for three hours.

Table 2

Solution	concentration 10^{-3}mol	α	pH	K	pK
A	2.776	0.102	3.55	3.55×10^{-5}	4.45
B	16.790	0.095	2.80	1.64×10^{-4}	3.78

The titration curve of the "A" solution is shown in Fig. 12. As the figure shows the titration curve of alginic acid solution is very characteristic. This fact would be due to the colloidal effect of high molecular electrolyte or due to the existence of carboxyl radicals having various dissociations, although that point needs further investigation. For that very reason, the ionization constant described in Table 2 means only the average ionization constant of saturated alginic acid solution. At all events, it may be said that the alginic acid is a fairly weak acid. Alginic acid is rather dissoluble in water, that is to say, about 0.3 g is dissolved in 100 cc of water when boiled for three hours, and thus obtained concentrated solution is not transparent but a protein-solution-like opalesque material.

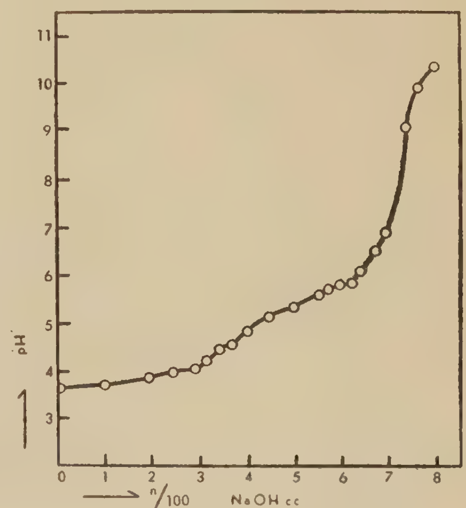


Fig. 12. Titration curve of saturated alginic acid solution "A", with n/100 NaOH

(iii) *Dissolution of alginic acid by cation exchange reaction*

Since the phenomenon of ion exchange may take place under certain conditions in all ionic solids, an investigation with regard to dissolution of solid alginic acid was attempted because the alginic acid would be dissolved into soluble alginate if cation exchange takes place between the acid and certain electrolytes whose cations are alkali metals, magnesium or ammonium radical.

Alginic acid is a rather weak acid as noted above, but sometimes it bahaves like a strong acid as follows: Alginic acid dissolves into soluble alginate when it reacts with many sorts of salts of rather strong acid through double decomposition as described in a previous paper[28]; also it liberates iodine from the mixed solution of potassium iodide and sodium nitrite as a strong acid does and it liberates sulfur from the solution of sodium thiosulfate too. The above facts may be explained as due to the cation exchange which takes place between hydrogen ion of carboxylic radical of alginic acid

and the cation of the salts.

Corresponding acid of the salt should be produced if the cation exchange reaction would take place between alginic acid and a salt following the reaction (2) :

$$\text{Alg-COOH} + \text{KA} \rightleftarrows \text{Alg-COOK} + \text{HA} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where, "K" represents a cation, and "A" an anion.

A preliminary examination of the above mentioned theory was made as follows : Alginic acid 0.2 g and the equivalent amount of various salts were put into 100 cc of distilled water and the change of pH values were measured after the passage of one hour ; pH value without salt was 4.5. The several results are shown as Table 3.

Table 3

Salt	pH	Remarks
CH_3COONa	4.2	Alginic acid is dissolved entirely within half an hour.
Na_2HPO_4*	4.4	Alginic acid is dispersed into small particles and partly dissolved.
$C_4H_4O_4Na_2$*	4.1	Same as above
NaF	4.0	Almost insoluble
HCOONa*	4.1	Alginic acid is dispersed into small particles and partly dissolved.
$C_4H_4O_6KNa$	4.0	Alginic acid is dissolved entirely within half an hour.
K_2CrO_4*	4.4	Alginic acid is dispersed into very small particles and slightly dissolved.
$C_2O_4Na_2$*	3.8	Alginic acid is dispersed into very small particles and partly dissolved.
$NaAsO_2$	4.4	Almost dissolved
$Na_2B_4O_7$	4.8	Alginic acid is dissolved entirely within half an hour.
NaCl	3.3	Apparently insoluble
KCl	3.4	Same as above

* See text

The mixed solutions asterisked in the table do not form true transparent colloidal solutions because they retain small particles of algin. However, they precipitate fibrous alginic acid gel by the addition of hydrochloric acid as usual soluble alginate solutions do. If the cation exchange proceeds as an almost or completely irreversible reaction, the dissolution of algin would take place, and if the reaction is almost or partly reversible, such dissolution would not take place, though alginic acid or algin with its cation replaced would be dispersed into small particles. That is to say, the ion exchange reaction between hydrogen ion of carboxylic radical of alginic acid and the cation of salt proceeds until equilibrium is reached.

The cation exchange equilibrium between alginic acid and NaCl or KCl was studied as follows : The cation exchange equilibria may be shown repsectively by the following formulas, (3) and (4).

$$\text{Alg-COOH} + \text{NaCl} \rightleftarrows \text{Alg-COONa} + \text{HCl} \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

$$\text{Alg-COOH} + \text{KCl} \rightleftarrows \text{Alg-COOK} + \text{HCl} \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Since hydrochloric acid ought to be produced if the above-mentioned reactions take place, hydrochloric acid was determined by neutralization with sodium hydroxide or by titration of Cl ion. Two-tenth gram carefully purified alginic acid powder prepared after the author's method, was put into conical flask together with the equivalent amount of NaCl or KCl, and 20–80 cc of redistilled water was added. After standing for 20 hours, the cation exchange equilibrium was determined by the following two methods : A) The equilibrium mixtures themselves were distilled by steam until the distillate amounted to 400 cc, and the distillate were titrated with n/40 NaOH solution to determine acidity ; on the other hand Cl ion of the distillate was determined by titrating with n/100 $AgNO_3$ solution after Mohr's usual method. B) After removal of matter left filtration, acidity and Cl ion of this filtrate were determined in the same way as above.

The purposes of these two experiments were as follows : Experiment A aimed to learn whether the titratable acid was truly hydrochloric acid or not. Experiment B was done to calculate the amount of absorbed electrolyte. The absorbed electrolyte was then calculated by subtracting titrated Cl ion of filtrate for added total Cl ion. The results are illustrated in Figs. 13, 14 and 15.

It may be said that the produced acid is hydrochloric acid on the ground that the titrated acidity agrees with Cl ion content of the distillate as shown in Fig. 13, though the amount of distilled acid is less than that obtained directly from the filtrate as is shown in Fig. 14. Under the experimental conditions, H ion of alginic acid exchanged with Na or K ion of NaCl or KCl though in small amount. That is to say, in NaCl solution about 0.045 to 0.08 mol of H ion of alginic acid is replaced by Na ion for 1 mol of alginic acid by cation exchange, and in KCl solution about 0.03 to 0.04 mol is replaced. The absorbed amount of electrolyte differs in the two cases, NaCl and KCl, as shown in Figs. 14 and 15. In NaCl solution, alginic acid scarcely absorbs the salt, whilst it does in KCl solution to as much as 0.48 to 0.55 mol for 1 mol of the acid.

2. Dissolution of Calcium Alginate

(i) *Preparation of calcium alginate*

MIWA[15] investigated some properties of calcium alginate prepared from *Undaria pinnatifida*. The calcium content in the calcium alginate prepared by him was 9.5 per cent although the theoretical calcium content in calcium alginate calculated from the formula $C_6H_7O_6 \cdot 1/2Ca$ was 10.25 per cent. He also prepared calcium alginate adding excess amount of calcium chloride solution to sodium alginate and dialyzing the calcium alginate gel until the reaction of Cl ion had disappeared. Calcium alginate thus obtained contained 21 per cent Ca, but Cl ion reaction was not found in this calcium alginate. From the above mentioned fact, he suggested that calcium would be combined not only with carboxyl radical of alginic acid but with its OH groups too. He did not recognize any remarkable difference concerning the solubilities of both

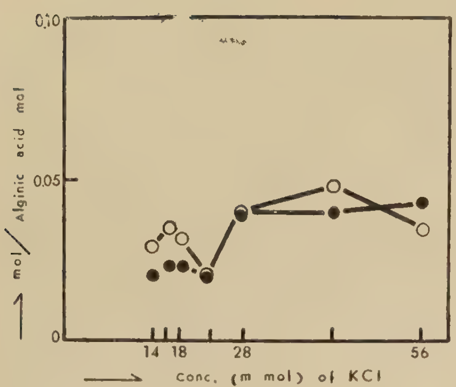


Fig. 13. Titrated acidity and chloride ion content of the distillate after method "A", where NaCl coexists with free alginic acid

○------ Titrated acidity
●------ Chloride ion content

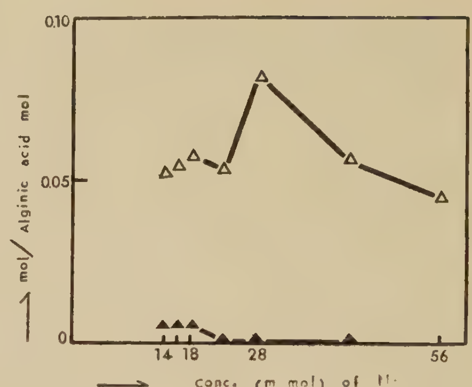


Fig. 14. Amounts of exchanged H ion and of the absorbed NaCl

△------ Amount of exchanged H ion
▲------ Amount of absorbed NaCl

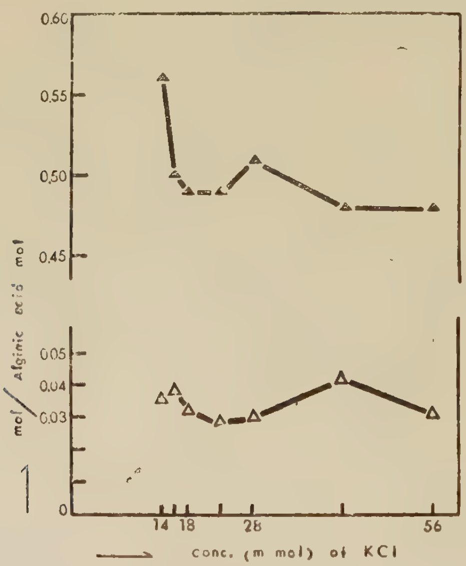


Fig. 15. Amounts of exchanged H ion and of the absorbed KCl

△------ Amount of exchanged H ion ▲------ Amount of absorbed KCl

preparates against sodium carbonate, sodium hydroxide and ammonium hydroxide solutions.

The author also obtained several preparates of calcium alginate containing calcium to as much as 7 to 20 per cent. They were prepared by adding various

amounts of 5 per cent calcium acetate solution to sodium alginate solution and dialyzing in distilled water until Ca ion reaction in dialyzing water had disappeared. The preparates thus obtained were dehydrated by ethanol and washed with ether and dried at 95°C. Since no remarkable difference was recognized concerning their solubilities as MIWA described in the range of calcium content 9.7 to 10.5 per cent, the preparates containing calcium in the range of 9.8 to 10.5 per cent were used for ascertaining the mechanism of manufacturing algin from calcium alginate.

(ii) *Dissolution of calcium alginate by cation exchange reaction*

The cation exchange reaction between calcium alginate and an electrolyte should take place following the formula (5).

$$\text{Ca-Alginate} + \text{KA} \rightleftarrows \text{K-Alginate} + \text{CaA} \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

where, "K" represents a cation, and "A" an anion.

Calcium alginate would be dissolved into soluble alginate, if the above described reaction (5) is almost or perfectly irreversible and "K" belongs to a certain alkali metal or to ammonium radical or to magnesium. Those KA were previously reported[19] as shown in Table 4.

Table 4

Na_2CO_3 ;	Na_2HPO_4 ;	Na_3PO_4 ;	$(COONa)_2$;	Na_2SO_3 ;	NaF ;	$Na_2S_2O_3$;
KIO_3 ;	$KSiO_3$;	Rochelle salt ;	$K_4Fe(CN)_6$;	etc.		

It is considered that the reaction (5) would be made irreversible if produced CaA is removed from the system of the reaction. There are two procedures by which that removal may be effected. One is as follows: Produced CaA is removed by precipitation because its solubility product is scanty. The other is as follows: Produced CaA is removed by the formation of a complex salt between CaA and KA; examples of this type are $Na_2S_2O_3$ and rochelle salt in the table. In the former case a turbid solution is obtained because of the precipitation of CaA and in the latter, on the contrary, a transparent solution is obtained because of the formation of a soluble complex salt. The dissolution would not take place if the reaction is almost or perfactly reversible even though "K" belongs one of alkali metals, ammonium radical or magnesium. The cation exchange equilibrium between calcium alginate and sodium chloride solution was investigated as follows.[24]

Portion of calcium alginate, each 0.7667 g as anhydride, were put into 100 cc of water, various amounts of sodium chloride were added and the mixtures were boiled for 30 minutes. After boiling, the mixtures were filtered and washed. The dissolved algin, calculated as sodium alginate, and Ca content were determined with the results shown in Table 5.

Because Ca content of the investigated calcium alginate was 79.74 mg, if the

Table 5

NaCl added (g)	0.10	0.20	0.30	0.50
Disssolved Ca (mg)	7.36	12.27	14.89	15.81
Dissolved algin (mg)	25.6	44.3	45.2	73.7

calcium alginate is converted into sodium alginate entirely by cation exchange, 774.5 mg of sodium alginate would be produced. When 0.50 g of NaCl was added, 19.8 per cent of Ca was dissolved and it was calculated that 9.5 per cent of calcium alginate was converted into sodium alginate in this condition. The reason why the dissolved Ca content is larger than the dissolved algin content which is calculated as sodium alginate, is considered to be that the dissolved Ca content involves both what is produced by cation exchange and what is combined with soluble algin.

It is well known that the inverse reaction takes place easily, that is to say, calcium alginate is ordinarily gelled out by adding excess amount of calcium chloride solution to sodium alginate solution. This fact is usually applied to obtain calcium alginate both in laboratory and in commercial practice. Calcium alginate gel thus obtained is very contracted, but the gel is remarkably swollen when it is treated with sodium chloride solution by the cation exchange reaction.

The cation exchange equilibrium (5) would proceed to produce sodium alginate as described above, if the produced calcium salt is removed from the system of the reaction by any procedure. The examples of those attempts are illustrated in formulas, (6) and (7), as follows :

$$\text{Ca-Alginate} + \text{Na-Acetate} \rightleftarrows \text{Na-Alginate} + \text{Ca-Acetate}$$
$$+\ (COONa)_2 \downarrow \quad \text{Ca-Oxalate} + \text{Na-Acetate} \quad \cdots\cdots (6)$$

$$\text{Ca-Alginate} + \text{NaCl} \rightleftarrows \text{Na-Alginate} + CaCl_2$$
$$+\ Na_2CO_3 \downarrow \quad CaCO_3 + \text{NaCl} \quad \cdots\cdots (7)$$

Algin may be manufactured with smaller amount of sodium oxalate or sodium carbonate by employing methods which follow the formulas above stated. The new recommendable process is described later applying a principle based upon the formula (7).

3. General Theory Concerning the Process of Algin Manufacture

(i) *General theory concerning the process of algin manufacture*

It has been mentioned above that alginic acid or insoluble alginates are dissolved into soluble alginate by cation exchange reaction. The general theory of the process of algin manufacture may be described as follows.

The cation exchange equilibrium between insoluble algin, such as alginic acid or insoluble alginate, and an electrolyte concerned with the dissolution of algin is presented in the following formula (8):

$$\text{Alg-COOX} + \text{MA} \rightleftarrows \text{Alg-COOM} + \text{XA} \quad \ldots\ldots\ldots\ldots \quad (8)$$

where "X" represents a cation which forms an insoluble algin combining with Alg-COO^-, such as H or Ca, and "M" a cation which forms a soluble algin combining with Alg-COO^-, such as an alkali metal, ammonium radical or magnesium, and "A" an anion.

An insoluble algin is dissolved into soluble algin when the above reaction (8) is almost or perfectly irreversible. That is to say, the dissolution of algin takes place in the special case of cation exchange reaction. Practical application of this phenomenon to algin mannufacture is described later.

(ii) *Dissolution by cation exchange reaction of insoluble algin*

Only a few data have been published regarding the cation exchange reaction of insoluble algin. SEIYAMA[29)] reported the effect of neutral salts on the dissociation of alginic acid. He concluded that the effect of neutral salts on that dissociation was dependent upon the ion exchange reaction. He also calculated the ion exchange equilibrium constant, and from the results of the calculation he reported that the ion exchange reaction did not occur quantitatively. He used sodium chloride solution and free alginic acid in his research and obtained the ion exchange equilibrium exponent pK_2 in the range of 1.5~3.0 for the concentration of alginic acid, as *-log* C, 2.0~4.5.

The author also obtained pK_2 in company with solubility, pH, α and pK values. The experiments were undertaken as described in this chapter keeping equilibrium of the reaction for 20 hours at 25°C, adding various amounts of NaCl or KCl. Results are shown in Table 6.

Table 6

Added electrolyte *-log*C		Solubility of alginic acid *-log*C	pH	α	pK	pK_2
without electrolyte*		2.93	4.45	0.029	6.00	—
NaCl	1.85	3.07	3.85	0.162	4.56	2.76
	1.55	2.95	3.80	0.140	4.59	3.15
	1.37	2.96	3.50	0.289	3.90	2.57
KCl	1.85	3.10	3.75	0.222	4.30	2.53
	1.55	3.09	3.75	0.212	4.31	2.85
	1.37*	2.96	3.15	0.648	2.89	1.40

The titration curves of the equilibrium solutions denoted with asterisks in the table are shown in Figs. 16 and 17. The curves were obtained by titrating with n/100 NaOH after removal of the matter left after filtration. It is discussed through above mentioned results as follows.

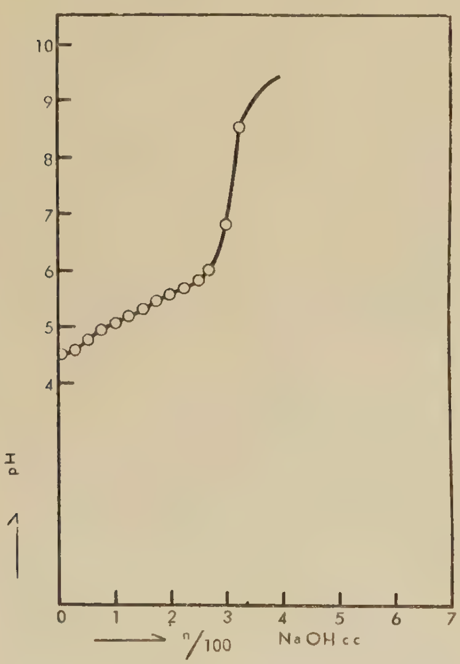


Fig. 16. Titration curve of saturated free alginic acid solution with n/100 NaOH allowed to stand for 20 hours at 25°C

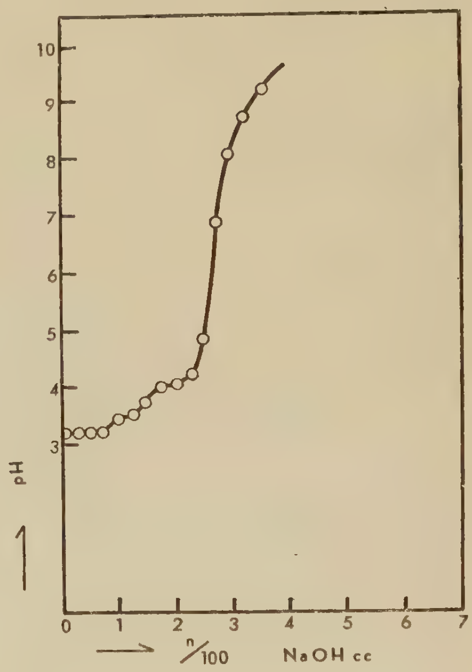


Fig. 17. Titration curve of saturated free alginic acid solution, where KCl coexists with the acid, allowed to stand for 20 hours at 25°C

The dissociation degree of the equilibrium solution is remarkably increased by addition of electrolyte, especially of KCl. This fact shows that the hydrogen ion of alginic acid was released to the solution as is shown by the decrease of pH. The author has calculated the cation exchange equilibrium exponent after SEIYAMA's equation, although the mechanism of ion exchange of alginic acid is considered very complicated and it should be further studied how to present the exponent. At all events, it may be said that the cation exchange reaction between alginic acid and electrolyte, NaCl or KCl, has taken place because pH, pK and pK_2 decrease and the dissociation degree of the solution is increased by addition of the electrolyte. It is then considered that the cation exchange reaction between free alginic acid and the salt of a weaker acid easily takes place and the alginic acid is dissolved into soluble alginate, though the reaction between free alginic acid and the salt of strong acid such as sodium chloride or

potassium chloride proceeds very slightly.

It is already noted above that the titration curve of saturated alginic acid solution was very characteristic and the reason for this fact was considered to be the existence of carboxyl radicals having various dissociation constants or the certain colloidal effects. The characteristic of the titration curve did not disappear by adding electrolyte to such a dilute alginic acid solution as shown in Fig. 16 or 17 and, therefore, the existence of carboxyl radicals having different dissociations is considered to be fairly possible although further investigations are needed because the colloidal effect would be eliminated by adding a fairly large amount of electrolyte to a dilute alginic acid solution.

Recently, results of researches on absorption of electrolyte by alginate were reported by WASSERMANN and his collaborators[30-32] and it was referred to the cation exchange of insoluble alginate which is concerned with the absorption of electrolyte by fully-swollen alginate fibres.

They reported in a series of papers that a curling up of stretched, chain like, giant molecules, and the accompanying macroscopic effects, could be brought about by stoichiometrically well-defined simple metatheses. The papers dealt with the preparation of fully-swollen, cylindrical alginic acid and alginate gels and with the absorption of simple electrolytes by these materials, but no attention was paid to the matter of algin manufacture.

As already mentioned, algin was first extracted by STANFORD[1] from a seaweed with sodium carbonate and since then a few works on algin manufacture have appeared. DILLON[2] proposed to use ammonia, and TAKAHASHI[10] devised a new method for algin manufacture in which he digested with ammonium oxalate. However there are no other available works on manufacturing algin from brown algae.

As is described in this paper, it is established that many sorts of electrolytes can be used for algin manufacture after a proper pretreatment.

III. NEW RECOMMENDED PROCESSES FOR ALGIN MANUFACTURE

Since algin occurs in various combined forms in brown algae as has been described above, it is desirable to pretreat algae with some certain proper reagent solution in order to convert the algin into a definite form, because algin can be extracted by suitable manufacturing process according to the pretreatment. The author discovered that many sorts of electrolytes are available for the extraction of algin from brown algae as described above. From among them two recommendable processes are described in this chapter.

1. Algin Manufacture from Brown Algae Macerated with Sodium Sulfite

It has been customary pretreat brown algae with dilute hydrochloric acid solution. This treatment is used to make algin easily dissoluble in actual practice and to extract potassium salts, iodine, mannit and pigment or other impurities, although little consideration has been given to converting algin into free alginic acid.

Since it is considered that algin in brown algae is converted almost entirely into free alginic acid by pretreating with dilute hydrochloric acid, tests have been conducted to ascertain whether that theory (p. 110) is applied in practice or not. The electrolytes shown in Table 7 have usable for manufacturing algin from brown algae which had been pretreated with dilute hydrochloric acid solution and the applicablity of the theory was ascertained as described below :

Table 7

M_3PO_4 ;	M_2HPO_4 ;	MH_2PO_4 ;	M_2SO_3 ;	$MHSO_3$;	$M_2S_2O_3$;	Rochelle salt ;
MNO_2 ;	HCOOM ;	CH_3COOM ;	$M_2B_4O_7$;	M_2CO_3 ;	$MHCO_3$;	MOH ;
M_2S_{2+x} ;	MBO_3 ;	M_2CrO_4 ;	M_2O_2 ;	MCN ;	etc.	

where "M" represents a cation, alkali metal, ammonium radical or magnesium.

It was observed that there are two cases of dissolusion of alginic acid with the electrolytes shown in the table. One is the case that the reaction proceeds keeping acid, and the other is the case that the reaction proceeds alkaline. The reason is obvious that the reaction (2) proceeds keeping acid when "A" is an acid radical, while the reaction proceeds keeping alkaline when "A" is OH^- or CO_3^{--}. Since the latter represents the case of dissolution by neutralization, it may be called a peculiar case of cation excange reaction which is perfectly irreversible. That is to say, including the dissolution by neutralization, the dissolution of alginic acid by electrolytes takes place through cation exchange reaction.

Among the electrolytes enumerated in the table, Na_2SO_3 is a very highly recommendable one, because the macerated mass or diluted solution is restrained from decomposition by microorganism and algin is bleached by SO_2 which is generated in the process of the reaction (9) :

$$2\text{Alg-COOH} + Na_2SO_3 = 2\text{Na-Alginate} + H_2O + SO_2 \quad \ldots\ldots\ldots\ldots\ldots \quad (9)$$

Furthermore, the reaction is almost perfectly irreversible because the produced SO_2 would escape by heating of the system.

A practical method of manufacturing algin from brown algae with sodium sulfite is briefly described as follows.

In order to convert various types of algin in brown algae into free alginic acid, the algae are treated with 0.1 n hydrochloric acid solution for 15–30 minutes at 50–60°C.

After removal of acid solution, 15 per cent of sodium sulfite by weight to dried algae and 30 times that weight of water are added to the remaining algae and heated for an hour at 60–70°C, then the bodies of the plants are completely disintegrated. Further treatment may be performed as in an ordinary method.

2. Algin Manufacture from Brown Algae Macerated with Sodium Chloride and Sodium Carbonate ....(SUZUKI'S Method)

The above described theory, in Chapter II, was ascertained as applicable practically for manufacturing algin from brown algae which are pretreated by calcium chloride solution in order to convert algin in the plant body into calcium alginate, and a new recommendable process was established by the author.[24]

The process is shown in diagrammatically in Fig. 18. The author proposes to name this "SUZUKI's Method" which was established by fundamental investigation briefly recounted as follows.

The investigation was made with *Laminaria angustata*. The material was first shattered to small pieces, about 5×5 mm in size. After submitting these pieces successively to each treatment described below, frozen microtome sections of them were stained with one per cent methylene blue solution in alcohol and examined under microscope. The results are shown in Figs. 19–31. The structure of the initial material is shown in Figs. 19 and 23. The process of each treatment is described as follows.

(i) *Swelling with water*

The material was treated with boiling water for 30 minutes in order to induce swelling of the tissue and to extract soluble matters, such as potassium chloride, iodine, mannit or impurities. The structure of the material thus treated is shown in Figs. 20 and 24. The parenchymatous cells are rather contracted by this treatment although the sections are swollen as a whole. It is considered that insoluble alginate in the walls of the parenchymatous cells, such as calcium alginate is contracted by boiling in water as described above.

(ii) *Steeping in calcium chloride solution*

After the conclusion of the preceding treatment, the water was drained, and the material was treated with 0.5 per cent calcium chloride solution at 100°C for 30 minutes. The structure of the material thus treated is shown in Figs. 21 and 25. Algin in the cell walls would be converted into calcium alginate almost entirely. The plant tissues are obviously contracted by this treatment and the epidermal tissues are made so firm by calcium chloride solution that they would not be destroyed by further treatment with either sodium chloride or sodium carbonate solution as described later.

(ii) *First digestion with sodium chloride solution*

The liquor used for the second treatment was thrown away, then the material was washed with water and was treated with 0.5 per cent sodium chloride solution at 100°C

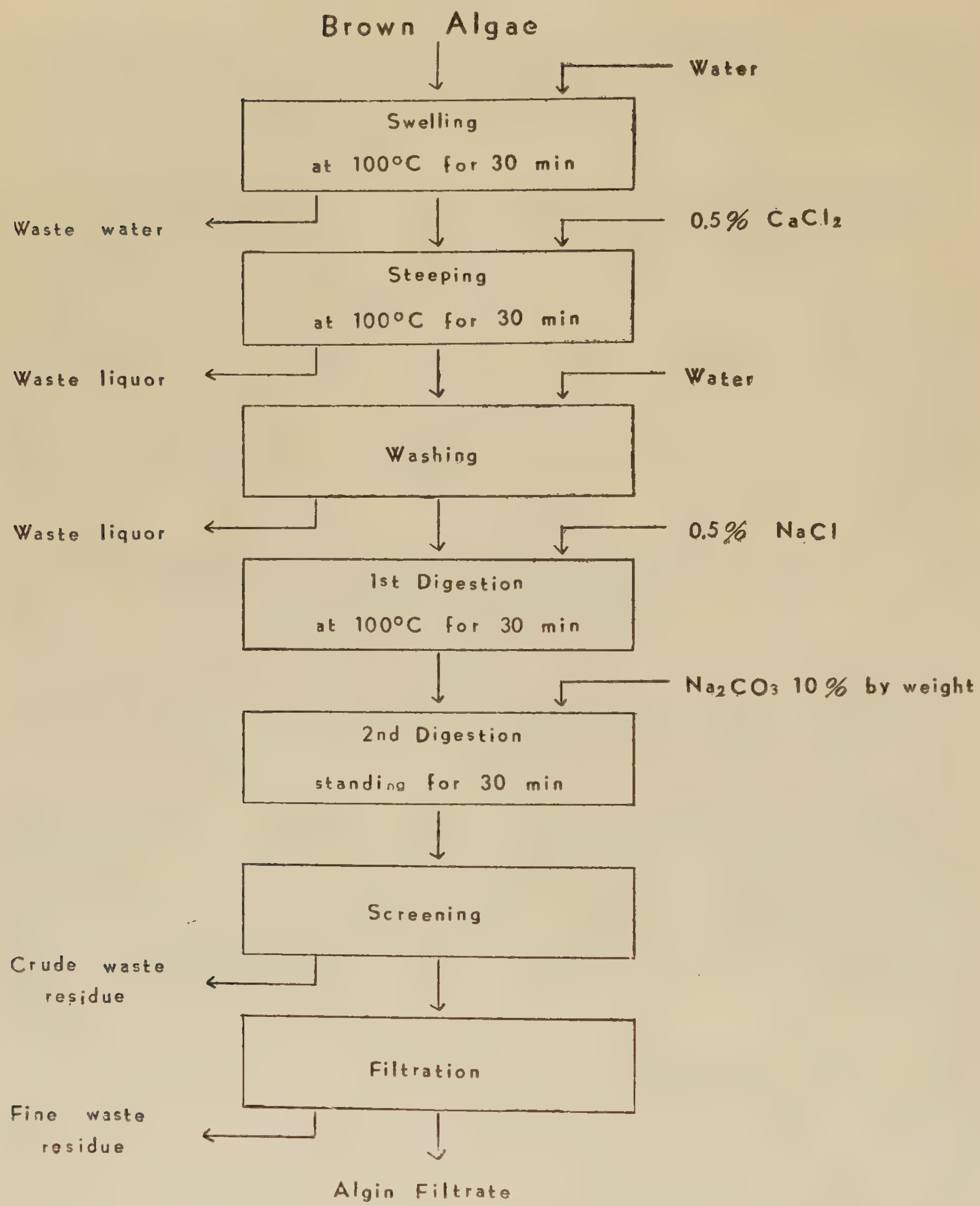


Fig. 18. Diagrammatical illustration of Suzuki's method

for 30 minutes. The tissues became greatly swollen and the cell walls of the inner parenchymatous tissue were partly destroyed. However, the sections continued to maintain their original shapes as a whole as shown in Fig. 22. The reason of this may be explained by above mentioned cation exchange reaction. That is to say, calcium alginate in the cell walls is partly dissolved out from the cells, while the epidermal

cells are made firm by the previous treatment with calcium chloride solution. The epidermal cells do not suffer damage by present treatment whilst viscous algin solution is dissolved out from the material.

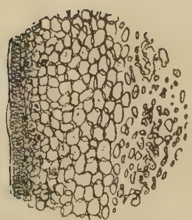
(iv) *Second digestion with sodium carbonate*

The liquor used for preceding treatment was not cast away this time, but 0.1 g of sodium carbonate was added to the digesting mixture at 90°C and the mixture was allowed to stand for 30 minutes. The results are shown in Figs. 27–31. The epidermal tissue suffers hardly any damage by the present treatment as shown in the figures while algin in parenchymatous and filamentous tissues is dissolved. The reason why the epidermal tissue does not suffer damage by treatment with sodium carbonate is considered to lie in the fact that the algin is preliminarily dissolved in part and the algin is well swollen by treating with sodium chloride solution; algin is very easily dissolved by sodium carbonate solution within a short time, because the epidermal tissue is not destroyed under such a mild contact with sodium carbonate.

The same treatments as described above were carried through with *Laminaria longissima* and *L. ochotensis*; the same results as in the case of *L. angustata* were attained also with these species.

Since it is proved that algin can be manufactured by the above described procedure without destroying epidermal tissue and hence without dissolving out the pigment from the assimilatory cells, a practically recommendable method for algin manufacture is developed as follows.

Treat the dried brown algae with boiling water for 15–30 minutes to swell the plant body and remove soluble matters and other impurities. Drain the water, and treat the algae with 0.5 per cent calcium chloride or saturated calcium hydroxide solution for 15–30 minutes at 80–100°C to convert algin in the cell walls into calcium alginate and to make the pigment in the epidermal cells insoluble. Cast away the liquor, and treat the algae with 10 per cent sodium chloride by weight to dried algae and 30 times that weight of water for 30–60 minutes at 80–100°C. Within this period calcium alginate in the cell walls is swollen by cation exchange producing soluble algin, while the pigment is retained in the plant body. Do not cast away the liquor; macerate the remaining bodies of the algae with sodium carbonate added to as large amount as 10 per cent by weight of the original dried algae for 10–20 minutes with gentle stirring. Allow to stand for several hours so as to make maceration complete; the pigment and the epidermal cells would remain undissolved and undamaged. The colour of the algin thus obtained is almost white without any bleaching measures and its viscosity is very high owing to the weak attack of alkali solution. The filtration is very easily performed because epidermis and mucilagenous substance excepting algin are scarcely dissolved.


Fig. 19

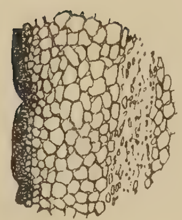

Fig. 21


Fig. 23

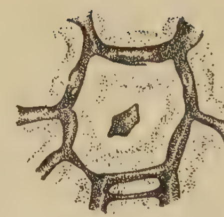
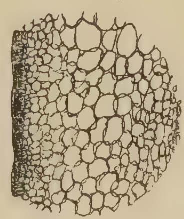
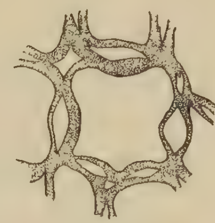

Fig. 25


Fig. 20


Fig. 22


Fig. 24

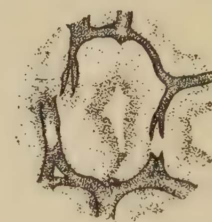

Fig. 26

Fig. 19. Section through a sample of non-treated material
Fig. 20. Section through a material treated with boiling water for 30 minutes
Fig. 21. Section through a material treated further with 0.5% $CaCl_2$ solution at 100°C for 30 minutes
Fig. 22. Section through a material treated further with 0.5% NaCl solution at 100°C for 30 minutes
Fig. 23. Part of the inner parenchymatous tissue in Fig. 19. Magnified
Fig. 24. Part of the inner parenchymatous tissue in Fig. 20. Magnified
Fig. 25. Part of the inner parenchymatous tissue in Fig. 21. Magnified
Fig. 26. Part of the inner parenchymatous tissue in Fig. 22. Magnified

IV. STUDIES ON PRETREATMENT OF ALGAE

It has been made clear that pretreatment of the brown algae for algin manufacture should be made to meet the following two purposes. One is to extract mineral salts, mannit or impurities, and the other is to convert algin in algae into a definite state of combination. To achieve these purposes, two kinds of pretreatments have often be combined, for example, the first treatment aiming at removal of soluble matter from the algae is followed by the second treatment aiming at conversion of algin in brown algae into a definite form of combination.

As for the pretreatment of brown algae, there have been published only a few reports. The above mentioned pretreatment methods[3)-6)] were originally proposed as a measure to obtain iodine from extracted liquor. According to TAKAHASHI[9)] dilute hydrochloric acid is the most suitable solution to pretreat algae in order to extract mineral salts and mannit but potassium salts are not so well extracted as the other soluble matters are. He suggested that potassium existed party combining with alginic acid.

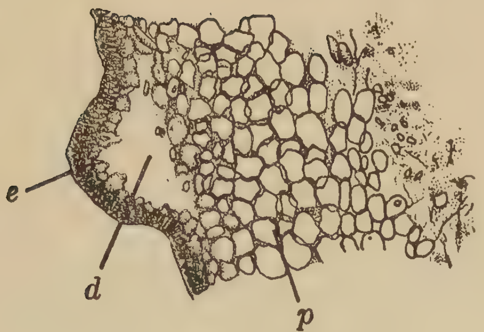


Fig. 27

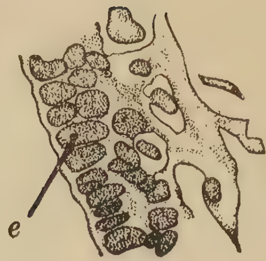


Fig. 28

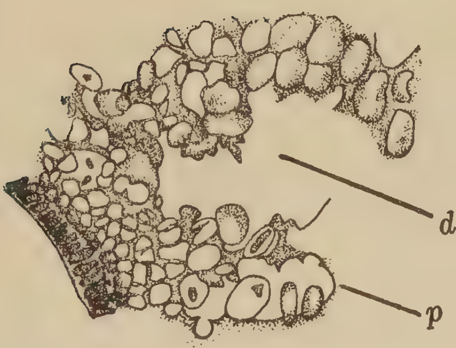


Fig. 29



Fig. 30



Fig. 31

Figs. 27–31. Section through the material treated further with 0.5% Na_2CO_3 at 90°C for 30 minutes

d, Damaged spaces due to the digestion of the tissue
e, Epidermal tissue, not damaged
f, Filamentous tissue, disintegrated with scattered residues
p, Parenchymatous tissue, half damaged

The author's own investigation on pretreatment of algae mainly with fresh materials was carried on as described below.

1. Experimental

To learn the swelling of the algal fronds when soaked in water or other solutions the following experiments were made.

(i) Ten grams of dried material and 50 g of wet material of *Laminaria japonica* were respectively placed in a beaker and 150 cc of boiled water was poured into it. The beaker was heated at the boiling point of the contained solution for 5–60 minutes and the water absorbed by the material was determined as shown in Table 8.

Table 8

Material	Duration of treatment (min)	Absorbed water to the material (%)
Wet	5	58
	10	44
	20	18
	30	26
	60	20
Dried	5	280
	10	258
	20	288
	30	336

(ii) Ten grams of dried materials of *Laminaria longissima* and *L. religiosa* respectively were treated with 300 cc of water at 100°C, with 300 cc of 0.1 n hydrochloric acid at 40°C and with 300 cc of 1 per cent calcium chloride solution at 60°C for 15 minutes and the absorption of treated liquor was determined as shown in Table 9.

Table 9

Material	Treatmont reagent	Swelling (%)
L. longissima	Water	380
	0.1 n HCl	50
	1% $CaCl_2$	340
L. religiosa	Water	310
	0.1 n HCl	30
	1% $CaCl_2$	340

From the results is known the fact that wet fresh algae do not absorb the liquor used for treatment so much as dried samples do, and the pretreatment with dilute hydrochloric acid results in minimum swelling of the dried material.

(iii) Wet materials of *Laminaria japonica* 200 g in weight, were placed in a beaker and 600 cc of water or either of the solutions mentioned below were added at 100°C and

boiled for 5 minutes, after which the soaked algae were taken away and fresh materials 200 g in weight, were put into the extract and extraction was repeated in the same way. The extraction was thus repeated from three to seven times, with precaution to keep the volume of the extract constantly at 600 cc by adding water or solution. For this treatment use was made of water or any one of the following solutions, viz., 0.1 n hydrochloric acid, 1 per cent calcium chloride solution, 0.005 per cent NaOH, 0.001 per cent NaOH, 0.01 per cent Na_2CO_3, sea water, or a mixture of fresh water and sea water. The results of three experiments which were performed independently of each other are shown respectively in Tables 10, 11 and 12.

Table 10

Treated agent	Number of times of treatment	Concentration of extract (%)			Recovery (%)		
		K_2O	Iodine	Mannit	K_2O	Iodine	Mannit
Water	1	0.319	0.012	0.027	88.2	58.5	37.8
	3	0.714	0.028	0.143	65.7	46.0	66.6
	5	1.100	0.079	0.271	60.8	76.1	75.9
	7	1.309	0.080	0.421	51.7	55.0	84.3
0.1 n HCl	1	0.311	0.009	0.068	85.9	43.8	95.2
	3	0.814	0.024	0.152	74.9	39.6	70.8
	5	1.106	0.044	0.243	61.1	36.3	68.0
	7	1.249	0.051	0.406	49.3	35.4	81.2
1% $CaCl_2$	1	0.334	0.020	0.053	92.5	98.7	74.2
	3	0.646	0.036	0.174	59.5	58.5	81.2
	5	1.149	0.076	0.286	63.5	73.2	80.1
	7	1.393	0.067	0.374	54.9	46.0	74.8

Table 11

Treated agent	Number of times of treatment	Concentration of extract (%)			Recovery (%)		
		K_2O	Iodine	Mannit	K_2O	Iodine	Mannit
Water	1	0.383	0.005	0.050	80.2	100.0	45.8
	3	0.914	0.010	0.299	64.2	66.8	63.9
	5	1.182	0.018	0.347	59.1	56.0	57.6
	7	1.279	0.025	0.395	39.0	—	51.4
0.001% NaOH	1	0.311	0.004	0.060	65.5	92.0	45.8
	3	0.900	0.010	0.248	63.2	66.8	55.7
	5	0.919	0.013	0.277	42.9	76.0	51.4
	7	1.254	0.023	0.416	37.7	60.0	45.4
0.005% NaOH	1	0.359	0.004	0.052	75.6	100.0	44.0
	3	0.993	0.010	0.190	46.7	66.8	87.6
	5	1.062	0.019	0.292	53.2	72.0	61.1
	7	1.120	0.021	0.361	33.7	57.2	49.6
0.01% Na_2CO_3	1	0.426	0.005	0.052	89.7	92.0	52.8
	3	0.890	0.010	0.218	62.5	66.8	72.7
	5	1.240	0.014	0.327	52.2	52.0	48.8
	7	1.350	0.036	0.409	40.6	65.6	52.3

Table 12

Treated agent	Number of times of treatment	Concentration of extract (%)			Recovery (%)		
		K_2O	Iodine	Mannit	K_2O	Iodine	Mannit
Water	1	0.239	0.035	0.080	71.9	93.0	50.9
	3	0.541	0.109	0.312	44.2	97.2	65.5
	5	0.880	0.087	0.455	53.0	46.7	57.5
	7	1.058	0.129	0.380	45.5	49.4	34.3
Sea water 1 : water 2	1	0.320	0.030	0.114	96.4	81.3	71.8
	3	0.550	0.081	0.435	65.1	71.7	91.4
	5	0.981	0.107	0.475	59.0	57.4	60.0
	7	1.172	0.140	0.517	50.4	53.1	46.5
Sea water 1 : water 1	1	0.320	0.038	0.072	96.4	100.0	45.6
	3	0.617	0.065	0.339	61.9	58.4	71.3
	5	0.947	0.087	0.558	57.0	46.7	70.3
	7	0.995	0.129	0.668	42.8	49.4	60.1
Sea water	1	0.296	0.035	0.055	89.2	93.0	35.2
	3	0.651	0.065	0.254	65.2	58.4	53.6
	5	1.077	0.118	0.414	64.8	63.2	44.4
	7	1.172	0.118	0.428	50.4	45.1	38.6

2. Discussion

(i) *Swelling of the algae*

The extractive power of a treating solution is greatly affected by its power to cause the algae to swell. It is proved that a dried algal material absorbs water to as much as about three to four times, and calcium chloride solution to as much as 2.5–3.5 times its own weight within the short duration of the treatment. However, a wet material does not absorb solutions above 50 per cent of its weight. Dilute hydrochloric acid is absorbed only as much as 50 per cent of the weight even by a dried algal material.

(ii) *Extractive power of the solution*

The curves of the increase in concentration of K_2O, iodine and mannit in the treated solution are shown in Figs. 32–42. From these figures it is known that the concentration of the extract does not increase linearly by the repetition of the treatmert in every case though concentration of the soluble matter is observed to a considerable extent.

The yield of the soluble mater by different treatments of the raw materials are shown in Figs. 43–51. From Figs. 43, 46 and 49 it is learned that each treatment shows remarkable difference of power to extract potssaium. In respect to the power of treating solutions to extract iodine some differences are shown amongst them and also there is fluctuation in some cases as shown in the figures. The minimum extractive power is observed in the case of the pretreatment with dilute hydrochloric acid solution and the maximum in the case of alkali treatment. The mannit is known to be less soluble than potassium chloride and iodine under the condition described above.

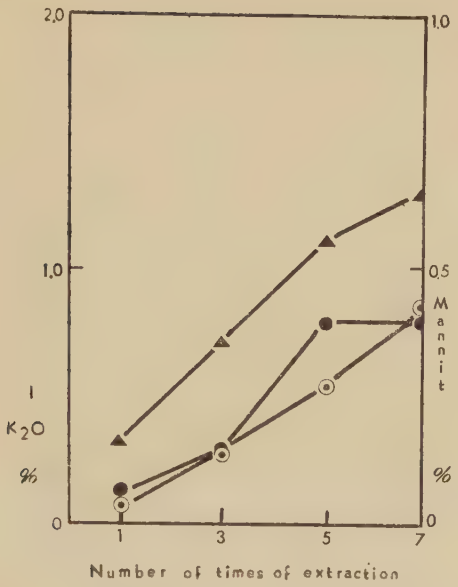


Fig. 32. Extracted with water

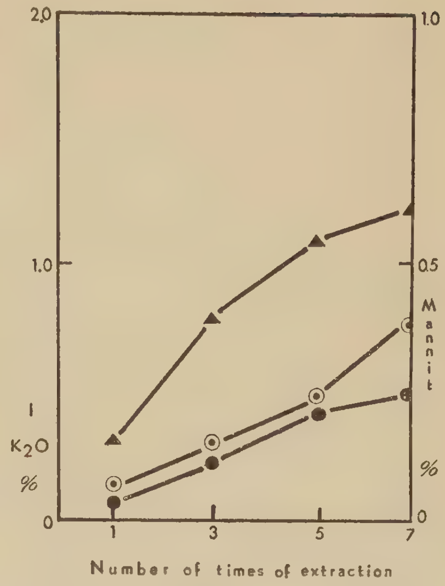


Fig. 33. Extracted with 0.1n HCl

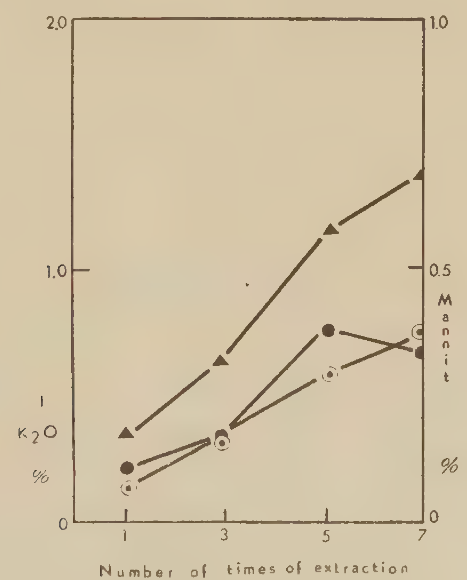


Fig. 34. Extracted with 1% $CaCl_2$

Figs. 32–34. Concentration of soluble matters in the algal extract (1)

▲------ Concentration of K_2O (%) ●------ Concentration of iodine (%×10)
⊙------ Concentration of mannit (%)

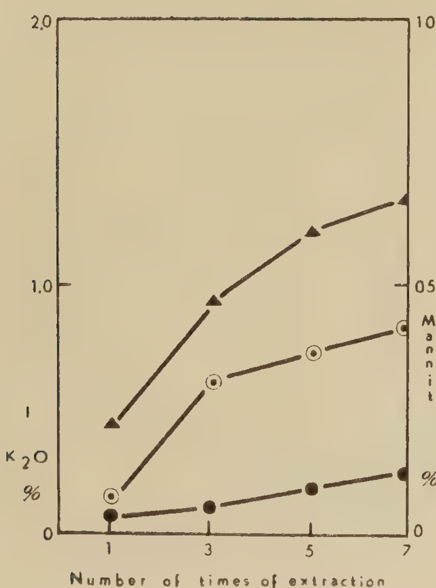


Fig. 35. Extracted with water

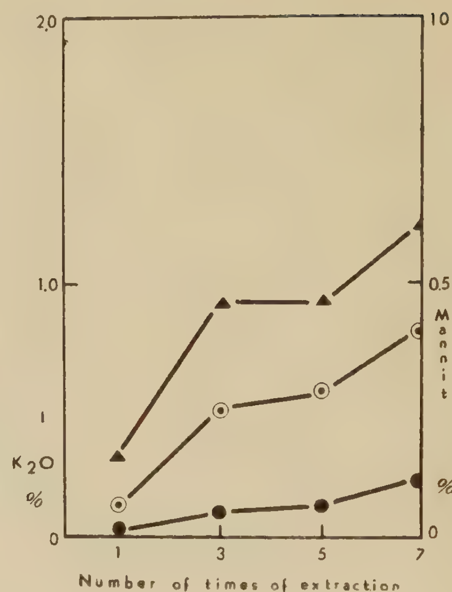


Fig. 36. Extracted with 0.001% NaOH

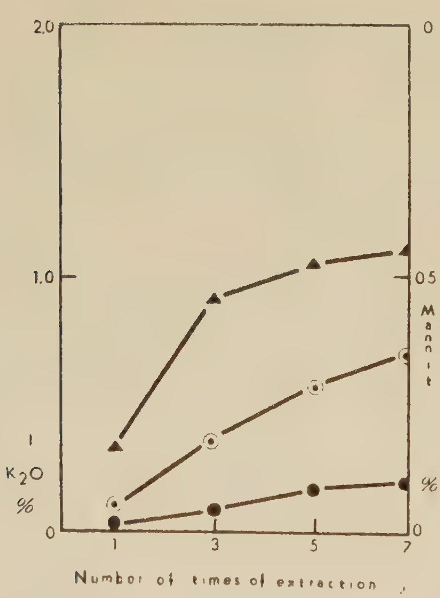


Fig. 37. Extracted with 0.005% NaOH

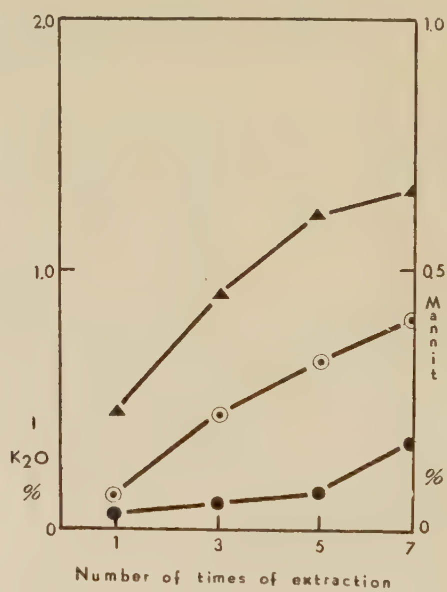


Fig. 38. Extracted with 0.01% Na_2CO_3

Fig. 35–38. Concentration of soluble matters in the algal extract (2)

▲------ Concentration of K_2O (%) ●------ Concentration of iodine (% × 10)
⊙------ Concentration of mannit (%)

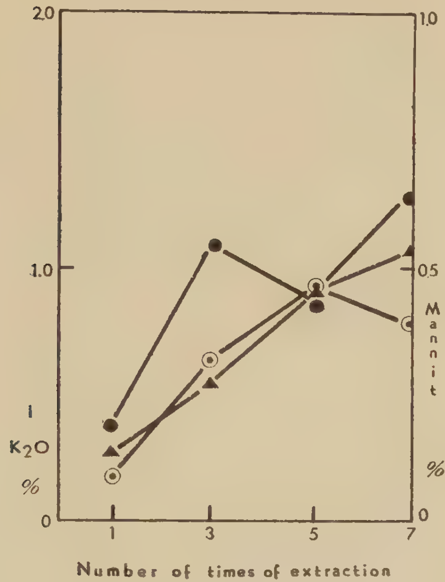


Fig. 39. Extracted with water

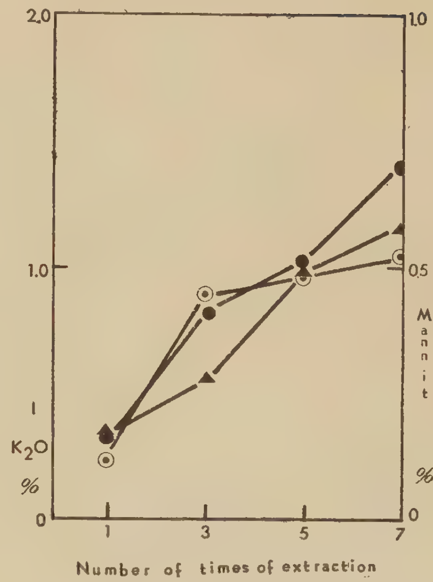


Fig. 40. Extracted with sea water 1 : water 2

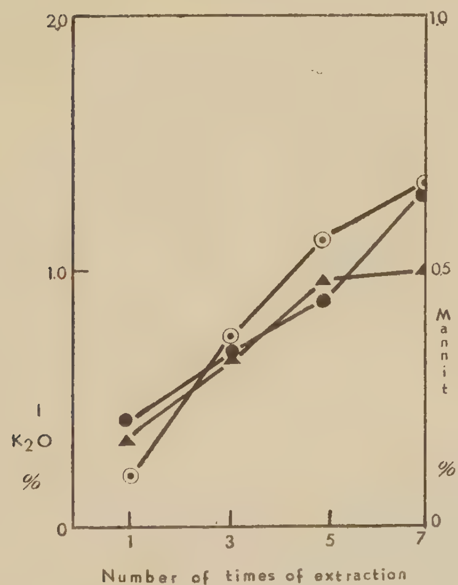


Fig. 41. Extracted with sea water 1 : water 1

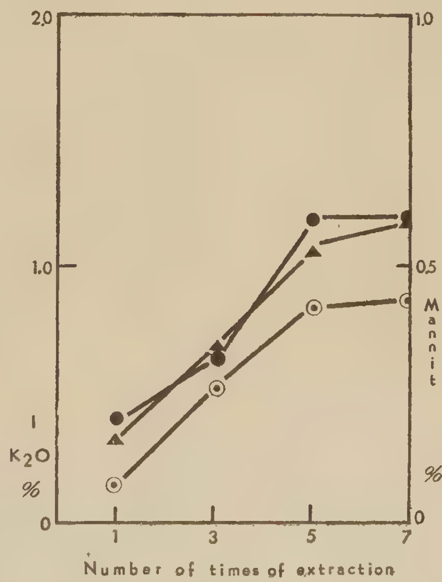


Fig. 42. Extracted with sea water

Figs. 39–42. Concentration of soluble matters in the algal extract (3)

▲------ Concentration of K_2O (%) ●------ Concentration of iodine (% × 10)
⊙------ Concentration of mannit (%)

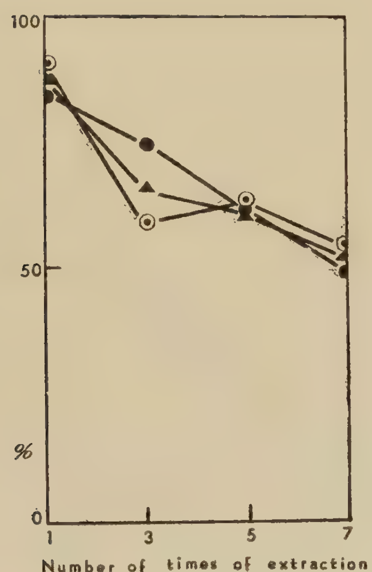


Fig. 43. Recovery of K_2O (%)

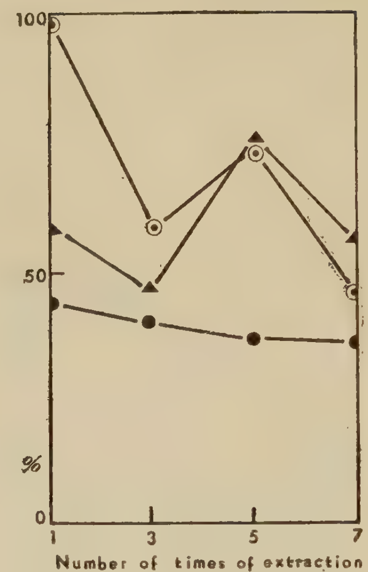


Fig. 44. Recovery of iodine (%)

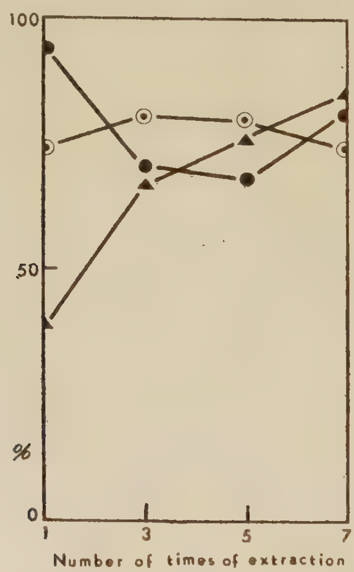


Fig. 45. Recvery of mannit (%)

Figs. 44–45. Recovery of soluble matters (1)

▲------ Extracted with water ●------ Extracted with 0.1n HCl
⦿------ Extracted with 1% $CaCl_2$

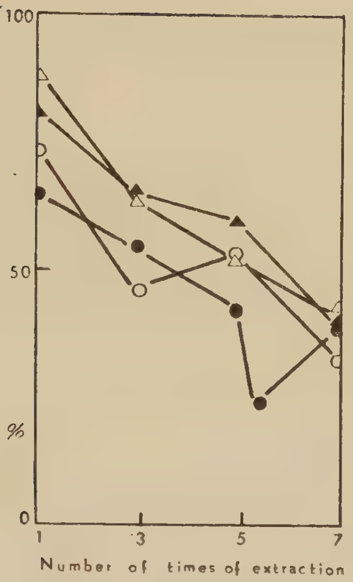


Fig. 46. Recovery of K_2O (%)

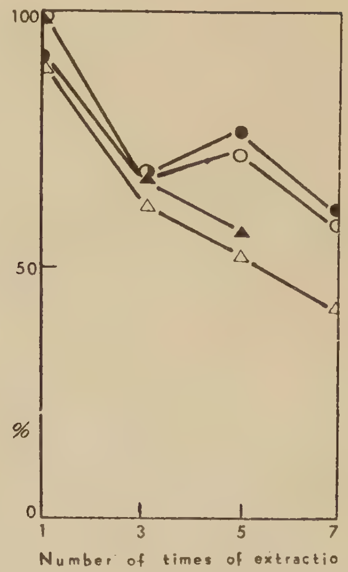


Fig. 47. Recovery of iodine (%)

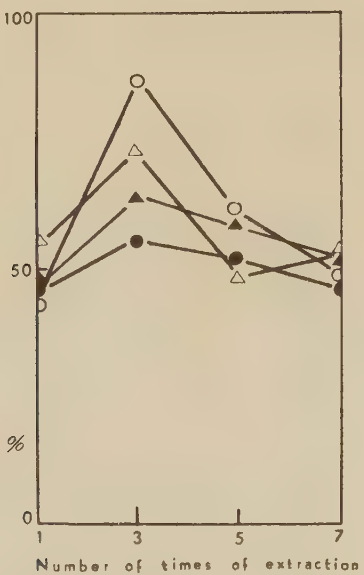


Fig. 48. Recovery of mannit (%)

Figs. 46–48. Recovery of soluble matters (2)

▲------ Extracted with water
●------ Extracted with 0.005% NaOH
△------ Extracted with 0.001% NaOH
○------ Extracted with 0.01% Na_2CO_3

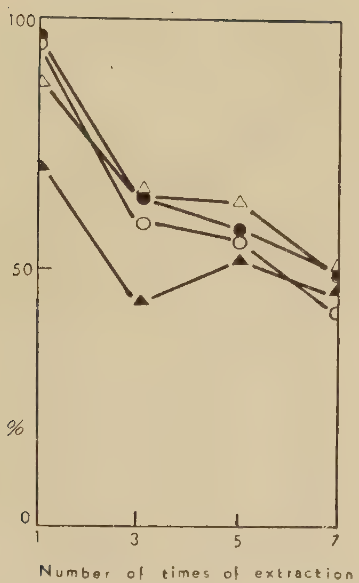


Fig. 49. Recovery of K_2O (%)

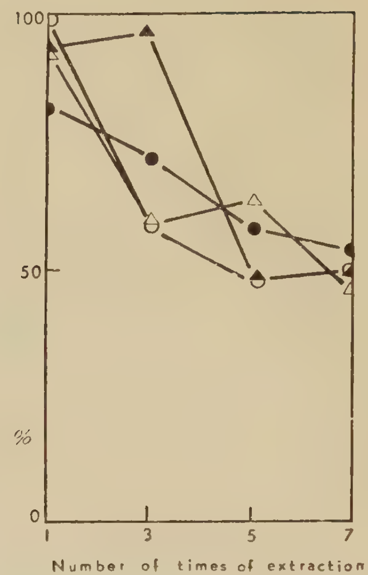


Fig. 50. Recovery of iodine (%)

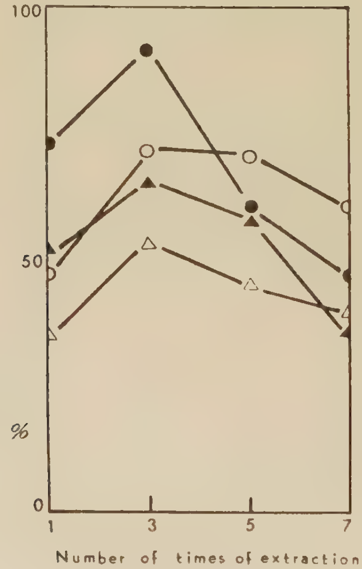


Fig. 51. Recovery of mannit (%)

Figs. 49–51. Recovery of soluble matters (3)

▲------ Extracted with water
●------ Extracted with sea water 1: water 2
○------ Extracted with sea water 1 : water 1
△------ Extracted with sea water

Since the concentrations of the extracts yielding by the first treatment are very low as shown in Tables 10-12 in every extraction, the repetition of the extraction is desirable in spite of the decrease of the yield. Sometimes a higher concentration of the extracts is required rather than a higher yield of the soluble matter because the recovery of the matter is fairy difficult and expensive when a dilute solution is used.

As no essential difference is found in the extractive powers of the examined pretreating solutions, the pretreatment of the algae for algin manufacture should be done taking into consideration mainly the conversion of the types of algin in the material, although the extractive power of the solution for pigment and other impurities needs consideration too.

V. RECOVERY OF MANNIT FROM BROWN ALGAE

Mannit has been obtained in laboratory from brown algae by extraction with hot methanol or ethanol and crystallization by cooling. But the solubility of mannit in these alcohols is very low, that is to say, 1.306 per cent in methanol and 0.757 per cent in ethanol even at their boiling points.[33] On account of this fact, these alcohols are not applicable industrially for the extraction of mannit. TAKAHASHI[34] reported a procedure to obtain mannit from algae by precipitating it as a copper compound from the dilute hydrochloric acid extract, but his method has not been applied industrially.

An attempt was made by the present author to recover mannit by means of fractional crystallization from other crystallizable matters, such as potassium salts or sodium salts, in the extract. Potassium and sodium chlorides are the most abundant salts in the extract of brown algae, and the solubility of potassium or sodium chloride in water does not vary greatly with the temperature while the solubility of mannit in the water varies extremely with the temperature. That is to say, nearly 70 per cent of mannit dissolves in water at 100°C, whilst it dissolves at the rate of only 10 per cent at 20°C.[33][34] An investigation was carried out to ascertain whether fractional crystallization of these substances in the extract could be done or not.

1. Experimental

The solubility of mannit in water has been reported as described above. Its solubility has been reported in concentration with the range of temperature from 20 to 100°C, but no report has appeared up to the present on the crystallization of dissolved mannit by cooling. As it was considered that the amount of crystallized mannit from the water solution by cooling did not agree always with the amount expected from its solubility, the following experiment was performed.

Experiment 1

Mannit was dissolved in water at 90°C so as get 30, 35, 45 and 50 per cent

solutions ; these solutions were allowed to stand for 24 hours at room temperature (20–30°C) and at 0°C. Recovered mannit was as presented respectively in percentages of the dissolved mannit in Table 13.

Table 13

Dissolved mannit (g/dl)	Recovered mannit (%) by cooling	
	20–30°C	0°C
30	24. 13	52. 03
35	31. 14	64. 17
45	52. 78	71. 32
50	57. 14	—

From the above result it is proved that mannit would be crystallized by cooling from its solution, although the mother liquor could not be separated from concentrated solution, such as 50 per cent solution, by cooling because mannit would be crystallized together with the mother liquor. It was experienced that mannit could not be separated from the mother liquor when 50 g of mannit was dissolved in 100 cc of water and the solution was cooled at 0°C.

Experiment 2

The excess of mannit, potassium chloride and sodium chloride were put into a tall flask to which was added 50 cc of water, and the mixture was placed in a thermostat with an accuracy of ±0.1°C. After the temperature of the mixture reached that of thermostat, the flask was shaken for 90 minutes at the rate of 150 times per minutes ; the experiment was operated at the range from –5°C to 80°C. After that the dissolved mannit, potassium chloride and sodium chloride were determined respectively. The determination was made as follows. The mixture was allowed to stand a short while at the experimented temperature, and then 20 cc of supernatant was pipetted into a measuring flask and the flask was filled up with water to 200 cc. Then the mixture was analyzed. A sample solution of lower temperature was taken from the flask and allowed to stand to reach room temperature, then it was pipetted for analysis. In order to avoid crystallization, a sample solution, which was treated under higher temperature, was taken from the flask, a known amount of water of the same temperature was added to it and it was pipetted after it reached the room temperature. The potassium chloride and sodium chloride used in this experiment were MERCK's reagents "for anlysis". The mannit used was obtained in the author's laboratory from *Laminaria* fronds extracting with ethanol and recrystallized several times from the ethanol solution ; its melting point was 160°C and its Cl content was lower than 0.0001 per cent.

The potassium chloride and sodium chloride content of the solution were calculated from the amounts of total ash and the chloride ion. The mannit content was

Table 14

Treating temperature °C	Dissolved amount (g/dl)				
	NaCl+KCl	Cl⁻	NaCl	KCl	Mannit
-5	31.15	16.69	22.52	9.13	7.89
0	32.48	18.35	22.29	10.21	10.25
20	34.18	19.11	21.95	12.23	12.65
40	35.13	19.59	22.15	12.98	19.14
60	30.13	16.88	19.57	10.56	28.07
80	25.63	14.11	15.31	10.32	41.08

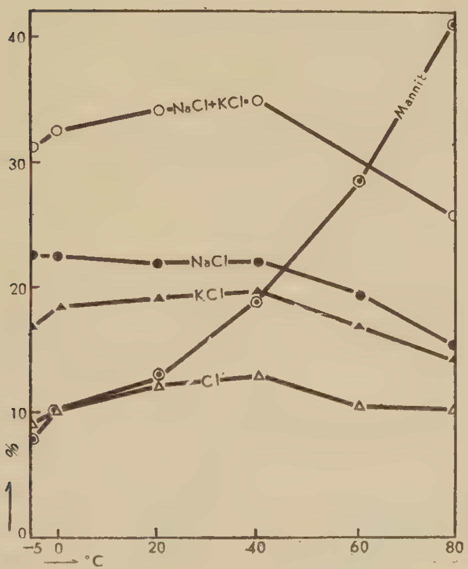


Fig. 52. Respective solubilities of mannit, potassium chloride and sodium chloride when these substances coexist together

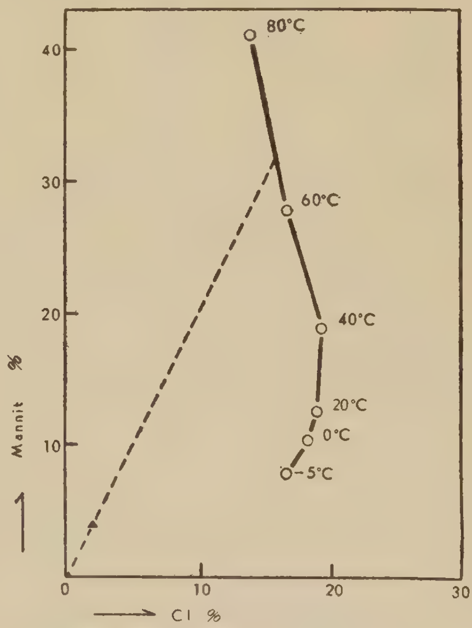


Fig. 53. Relation of solubilites between mannit and chlorides at each temperature, where chlorides are represented as Cl^-: dotted line; supposed line of evaporation of mannit being 4% and Cl^- 2%

determined by the author's method.[19] The results are shown in Table 14 and Fig. 52.

From the figure it will be noted that the solubility of mannit varies remarkably with the change of temperature and that the amount of potassium and sodium chloride may be represented by the amount of total chloride ion. The relation between the solubilities of mannit and total chloride ion is shown in Fig. 53. It may be observed from the figure what the saturation point of either mannit or salts of any solution is

reached when the solution is concentrated, and it may be determined which substance would be crystallized in given concentrating or cooling temperatures. For example, if the solution containing 4 per cent of mannit and 2 per cent of chlorides as Cl is evaporated at 80°C, chlorides would be saturated at the concentration of abont 16 per cent before mannit reaches its saturation as shown by the straight dotted line in the figure. By further evaporation chlorides would be crystallized and the evaporation would proceed along the curve, while mannit would not be crystallized till the evaporation attained the saturation point of the mannit, about 40 per cent, as shown in the figure. If the evaporation is stopped before the saturation point of mannit and the mother liquor is quenched, mannit would be crystallized with good yield but chloride would be crystallized quite sparingly.

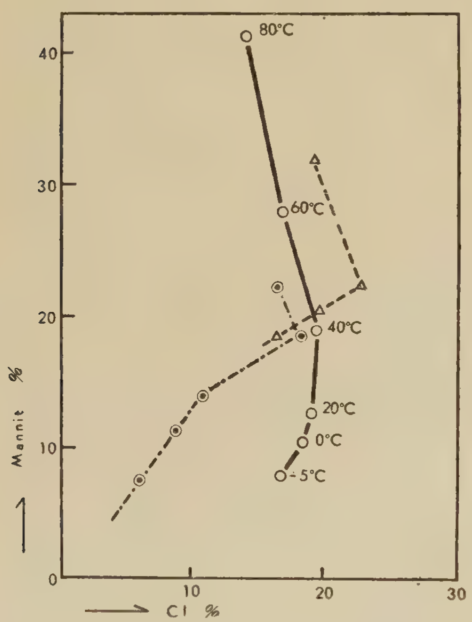


Fig. 54. Solubilities of mannit and chlorides at each temperature and actual concentration by evaporation of the algal extract, where chlorides are represented as Cl⁻

○------ Solubilities of each temperature
⊙ and △------ Stage of actual concentration by evaporation of algal extract

Experiment 3

Ten times by weight of 0.1 n hydrochloric acid was added to sample *Laminaria* fronds and boiled for 15 minutes. The extract was neutralized with sodium carbonate and filtered. The obtained liquor was evaporated at boiling point and determined amounts of mannit and chlorides were plotted in Fig. 54 showing two examples. From the figure it may be observed that the evaporation proceeds nearly along the curve after crystallization of chlorides takes place, although it does not proceed exactly on the curve. The reason for that may be the effect of other salts or organic matters existing in the extract.

2. Discussion

Fundamental experiments on the fractional crystallization of mannit from other dissolved matters in the extract of brown algae were made as explained above. It is suggested by the experiments that mannit can be obtained by fractional crystallization, but this can not be recommended as a fully suitable procedure to recover mannit from the extract.

VI. STUDIES ON THE PROCESS OF MANUFACTURING ALGIN

Works by previous investigators regarding the processes of manufacturing algin from brown algae are scanty. TAKAHASHI has published many papers on the subject, which form the principal part of his book entitled "SEAWEED INDUSTRY".[35] His works are all concerned with the manufacture of algin by sodium carbonate or ammonium oxalate. The present author has discovered that there are various processes of manufacturing algin from brown algae using many sorts of chemicals. The following investigations are concerned with new methods of algin manufacture.

As algin fairy unstable in its colloidal qualities, one should pay careful attention to this fact in every treatment throughout the process. In a factory it is required to manufacture algin having good qualities, that is to say, high viscosity, good yield, no colour and no turbidity. Since the requirements for these qualities are inconsistent with each other, the manufacture of algin is a very difficult undertaking in spite of the fairly simple process. If a higher yield is required, algin should be manufactured using excess of alkali and treating at higher temperature, but under such conditions the viscosity of algin would be decreased and the colour would be deep. The viscosity would also be decreased if bleaching is done in order to obtain colourless product. When the viscosity of the digested solution is high, it would be decreased again by long spells of filtration. In order to meet certain requirements in algin manufacture, one is compelled to sacrifice some others.

1. Temperature of Algin Treatments

As viscositiy of sodium alginate would be affected by the temperature of the treatments during the manufacturing process, some fundamental investigations were made as follows.[36]

Experimental and discussion

Alginate for experiment was prepared as follows:

(1) One hundred grams of dried *Costaria costata* were macerated with 3 litres of 2.0 per cent sodium carbonate solution at room temperature for 24 hours and the liquor was filtered after dilution with water. The residue was macerated again with 2.0 per cent sodium carbonate solution and the liquor was filtered. The two thus obtained filtrates were mixed and the liquor was poured into ethanol in order to obtain sodium alginate Thus obtained sodium alginate was washed with ethanol several times and next with ether, after which the preparation was dried in desiccator. The preparation is hereafter described as NaA.

(2) Maceration was made at 85–90°C for 2 hours and macerated mixture was allowed to stand for 24 hours. Other treatment was made in the same way as above. The preparation is hereafter described as NaB.

(3) Algin was prepared from the material macerated with 1.0 per cent ammonium oxalate in the same way as (1); the preparation is described hereafter as OxA.

(4) Algin was prepared from the material macerated with 1.0 per cent ammonium oxalate in the same way as (2); the preparation is hereafter described as OxB.

The yield and the specific viscosity of 0.2 per cent solution of these preparations are shown in Table 15.

The 0.2 per cent solution of each preparation was prepared and it was heated at 80°C and 95°C for 30–420 minutes. The specific viscosity of each solution at various stages of treatment was determined. The results are tabulated in Table 16, and illustrated in Figs. 55 and 56.

Table 15

Preparation	Yield (%)	Specific viscosity
NaA	14	7.36
NaB	19	8.64
OxA	15	6.50
OxB	25	9.45

Table 16 Change of specific viscosity by heating

Treated duration (min)	Preparation							
	NaA		NaB		OxA		OxB	
	Treated temperature (°C)							
	80	95	80	95	80	95	80	95
0	7.36	7.36	8.64	8.64	6.50	6.50	9.45	9.45
30	—	6.70	8.50	7.84	5.68	5.41	8.63	6.59
60	6.95	6.45	7.86	7.18	4.58	4.75	8.22	6.45
120	6.59	5.13	7.00	5.80	4.14	3.80	6.95	4.36
180	6.09	4.43	5.91	5.07	3.59	3.02	6.00	3.41
240	—	4.00	5.54	3.98	3.18	2.45	5.36	2.68
300	5.63	3.31	4.91	3.29	2.86	2.05	4.45	2.18
360	5.27	3.13	4.37	2.91	2.55	1.78	3.95	1.86
420	5.09	2.72	4.12	2.48	2.33	1.53	3.55	1.57

From these figures is assumed the following formula.

$$y = Ae^{-\alpha t} \qquad (10)$$

where, y represents specific viscosity at each stage of duration of heating, t represents duration of heating and α and A are the constants of the equation concerned with each preparation. Applicableness of the equation (10) is ascertained by means of Figs. 57 and 58. That is to say, the figures are constructed with $\log y$ at ordinate and with t at abscissa, and the erected line is drawn straight. The values of α are calculated and these values are compared for each preparation as shown in Table 17. It is shown in Figs. 57 and 58 that the viscosity of the alginate would be decreased by heating in

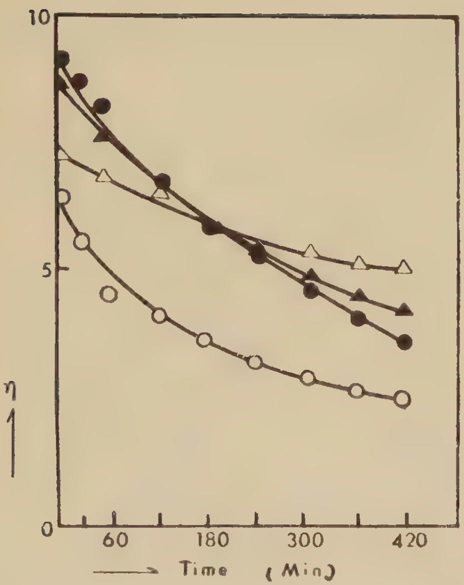


Fig. 55. Decrease in viscosity of algin solution after heating at 80°C

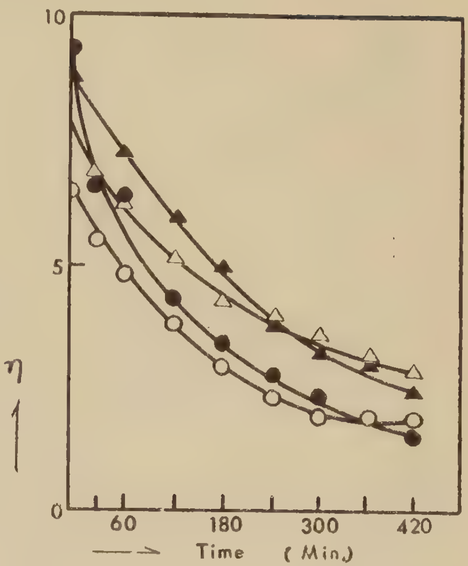


Fig. 56. Decrease in viscosity of algin solution after heating at 95°C

△------ NaA, ▲------ NaB,
○------ OxA, ●------ OxB,

accordance with equation (10). It is clear that the viscosity of algin would be decreased by heating, therefore long spells of heating should be avoided in every process of algin manufacture.

Table 17

Preparation	NaA		NaB		OxA		OxB	
Treatment temp. (°C)	80	95	80	95	80	95	80	95
Treatment duration (min)	30–420	30–420	30–420	30–420	60–420	30–420	30–420	120–420
α	0.06	0.17	0.13	0.24	0.17	0.32	0.17	0.35

2. Maceration of Algae

The yield, specific viscosity and colour tone of algin preparation and manufacturing processes were examined the material being macerated with several sorts of chemicals as follows.

(i) *Manufacturing processes with different pretreatments*

Since the yield, viscosity and colour tone of algin differ with the manufacturing

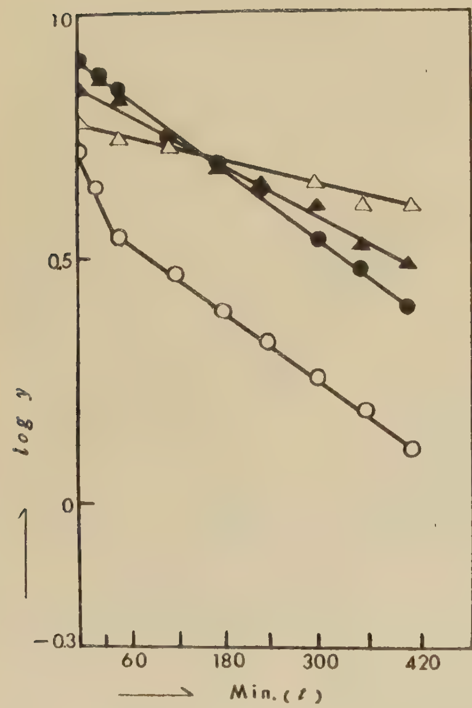


Fig. 57. The relation of *log y* to the duration of heating at 80°C

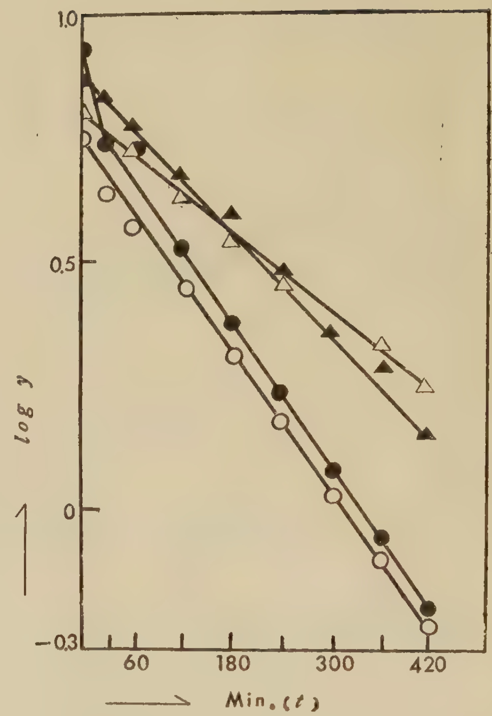


Fig. 58. The relation of *log y* to the duration of heating at 95°C

△------ NaA, ▲------ NaB,
○------ OxA, ●------ OxB,

procedures, the pretreatment process was first examined. Three kinds of pretreatment, namely, (a) pretreatment with water, (b) with calcium chloride and (c) with dilute hydrochloric acid, were compared with each other material being macerated with several chemicals.

These three kinds of preptreatment were carried out respectively as follows: Ten grams of dried *Laminaria longissima* were placed in a beaker and were pretreated (a) with 300 cc of water at boiling point for 15 minutes, (b) with 300 cc of 0.5 per cent calcium chloride solution at boiling point for 30 minutes, or (c) with 0.1 n hydrochloric acid at 50°C for 15 minutes respectively and separately. After the pretreatments, each of the materials was washed with water and macerated with Na_2CO_3, Na_2SO_3, $(COONH_4)_2$, Na_2HPO_4 or NaOH in 300 cc of water at 60°C for an hour with constant stirring. After maceration, 700 cc of water was added and the whole was filtered under reduced pressure. Alginic acid gel was then precipitated by adding 75 cc dilute hydrocholric acid (1 : 2). The water was separated with silk cloth, and collected gel was put into water at 55–58°C in order to dehydrate the absorbed water in the gel.

Thus obtained gel was washed with water of identical temperature and then with ethanol and ether, and dried in the desiccator. The yield, colour tone and specific viscosity of the algin were determined. The specific viscosity was determined with Ostwald's viscosimeter at 25°C, neutralizing alginic acid with sodium carbonate solution and adjusting the concentration of the solution at 0.2 per cent. The results are shown in Tables 18, 19 and 20.

Table 18. Yield(%) of alginic acid obtained by different processes

Maceration (g)		Pretreatment		
		Water	0.5% $CaCl_2$	0.1 n HCl
Na_2CO_3	2.0	21.2	22.2	20.0
Na_2SO_3	3.5	18.0	18.5	20.6
Na_2HPO_4	5.0	19.0	14.0	18.0
$(COONH_4)_2$	1.0	21.0	12.0	—
NaOH	1.0	4.2	2.0	19.0

Table 19. Specific viscosity of sodium alginate solution (0.2 %) obtained by different procedures

Maceration (g)		Pretreatment		
		Water	0.5% $CaCl_2$	0.1 n HCl
Na_2CO_3	2.0	69	59	103
Na_2SO_3	3.5	75	57	295
Na_2HPO_4	5.0	68	81	114
$(COONH_4)_2$	1.0	71	49	—
NaOH	1.0	54	—	109

Table 20. Colour tone of alginic acid obtained by different procedures

Maceration	Pretreatment		
	Water	0.5% $CaCl_2$	0.1 n HCl
Na_2CO_3	Pale brownish yellow	Brownish yellow	Pale yellowish brown
Na_2SO_3	Pale yellow	Pale brownish yellow	Pale brownish yellew
Na_2HPO_4	Greenish brown	Greenish brown	Pale yellow
$(COONH_4)_2$	Greenish brown	Greenish brown	—
NaOH	Greenish brown	Greenish brown	Pale yellowish brown

From the results the following discussion under three headings is suggested.

Yields :

In the case of pretreatment with water no remarkable difference of the yield can be observed as having connection with the maceration with examined chemicals excepting NaOH. In the case of pretreatment with dilute calcium chloride solution, Na_2CO_3 and Na_2SO_3 give larger yield than other chemicals, while NaOH gives the least yield. In the case of pretreatment with dilute hydrochloric acid solution, no remarkable

difference of yield is observed with the exception of the case of ammonium oxalate.

From the above mentioned facts, the following remarkable phenomena were noted: Sodium hydroxide solution macerates the algae well when the plants are pretreated with dilute hydrochloric acid solution while it hardly macerates the algae at all when the plants are pretreated with water or calcium chloride solution. These facts may be explained as follows: sodium hydroxide solution is able to dissolve free alginic acid into sodium alginate solution while it is scarcely able to dissolve calcium alginate or other insoluble algin in the algae. In other words, the cation exchange reaction between sodium hydroxide and free alginic acid is irreversible whilst the cation exchange reaction between sodium hydroxide and calcium alginate or other insoluble algin in the brown algae is nearly reversible. The practical examination agreed quite well in results with the fundamental experiments which are described above in Chapter II. Another remarkable phenomenon is observed in the case of the maceration with ammonium oxalate solution: it dissolves out algin from the algae when the plants are pretreated with water or calcium chloride solution but not when pretreated with dilute hydrochloric acid solution. This fact may be explained as follows: free alginic acid is not dissolved into colloidal solution by treatment with ammonium oxalate solution but it is dispersed into small particles as noted in Table 3 and for this reason algin can not be dissolved into the solution from the cell wall of the algae.

Viscosity:

The viscosity of sodium alginate obtained by pretreating the material with dilute hydrochloric acid solution is higher than that obtained with other solutions. The reason for this is considered to lie in the fairly mild conditions, under which the pretreatment with hydrochloric acid was done, namely in low concentration of the acid, 0.1 n, and at low temperature, 50°C for 15 minutes. Remarkable high viscosity is observed in the preparation which was pretreated with dilute hydrochloric acid solution and macerated with sodium sulfite solution. The reason may be as follows: the dissolution of algin would follow formula (9) and therefore the reaction would proceed in the weak acid and the decomposition of algin by bacteria would be prevented by the formation of sulfur dioxide during the maceration as described in Chapter III.

Colour tone of the preparatation:

The colour tone of alginic acid obtained by macerating the material with sodium sulfite is the faintest among the examined preparations. This fact may be explained as follows: sulfur dioxide produced according to formula (9) bleaches the algin preparation; sulfur dioxide would be liberated by the addition of hydrochloric acid to the macerated solution in order to precipitate alginic acid gel or it would be liberated in the macerating process when the pretreatment is made with dilute hydrochloric acid solution.

(ii) *Filtration in cases of different maceration*

Ten grams of *Laminaria longissima* were placed in a beaker and pretreated with

300 cc of water at boiling point for 15 minutes. After throwing the liquor away, residues were washed with water, and then 300 cc of water and various amount of several chemicals were added. The mixture was macerated at 60–70°C for an hour with continous stirring. After that 700 cc of water was added at 60°C in order to assist the filtration. Filtration of macerated mixture was made under reduced pressure till the filtrate amounted to 600 cc. After filtration 75 cc of dilute hydrochloric acid (1 : 2) was added to the filtrate in order to precipitate alginic acid gel. Precipitated gel was collected in a silk cloth and was put into water at 55–58°C in order to dehydrate the gel. Thus obtained wet gel was washed with water at the same temperature, then washed with ethanol and ether, and dried in the desiccator. Yield of alginic acid and the velocity of filtration were determined. The velocity of filtration was measured as the length of time needed for filtrating the macerated solution until 600 cc of filtrate was obtained. The results are shown in Table 21.

Table 21. Yield of alginic acid and the velocity of filtration, pretreating algal material with water

Maceration (g)		Yield of alginic acid (%)	Velocity of filtration (min/600 cc)	Specific viscosity of filtrate
Na_2CO_3	1.0	17.3	62	53.0
	2.0	21.0	51	51.6
	3.0	21.0	47	105.5
Na_2SO_3	2.5	15.0	57	52.3
	3.5	17.2	126	68.6
	4.5	18.2	83	77.7
$(COONH_4)_2$	0.3	3.0	16	18.5
	1.0	21.0	83	92.3
	2.0	21.0	75	114.5
Na_2HPO_4	3.0	15.3	44	48.9
	5.0	19.0	67	99.0
	7.0	20.0	59	109.2
NaOH	0.5	5.0	28	35.5
	1.0	5.0	20	63.3
	2.0	5.3	8	50.4

No remarkable differences concerning the maceration can be found when algae are pretreated with water, but NaOH can not macerate the algae well. It is considered that characteristic effects of the macerating chemical can not be found when the algae are pretreated with water because the algin in brown algae is not settled into a definite form.

The following experiments were carried out in order to learn the effect of the pretreatment with hydrochloric acid solution. Processes were almost the same as in the former experiments, except that the pretreatment was made with 150 cc of 0.1 n hydrochloric acid solution at 50°C for 15 minutes and that the material was *Laminaria religiosa*. The velocity of filtration was measured as the length of time needed for

filtration till 600 cc of filtrate was obtained. The results are shown in Table 22 and Fig. 59.

Table 22. Yield of alginic acid and the velocity of filtration, pretreating algal material with 0.1 n HCl

Maceration (g)		Yield of alginic acid (%)	Velocity of filtration (min/600 cc)	Specific viscosity of filtrate
Na_2CO_3	0.5	—	157	1.7
	1.0	17.3	346	33.6
	1.5	20.0	311	50.8
	2.0	21.0	325	54.7
	2.5	22.0	305	51.8
	3.0	21.0	333	59.3
	3.5	23.2	270	58.3
	4.0	23.0	305	46.9
Na_2SO_3	1.0	—	47	1.2
	1.5	—	38	1.1
	2.0	—	48	3.6
	2.5	15.0	62	7.7
	3.0	14.2	125	11.8
	3.5	17.2	90	19.0
	4.0	17.0	115	25.7
	4.5	18.2	61	10.0
	5.0	19.0	—	—

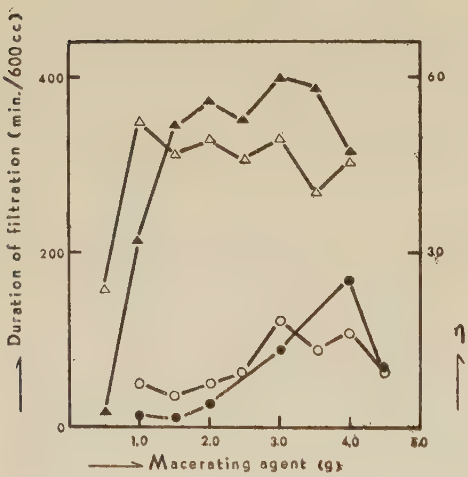


Fig. 59. Duration of filtration (min/600 cc) and specific viscosity of filtrate macerated with Na_2CO_3 and Na_2SO_3

△------ Duration of filtration in the case of macerating with Na_2CO_3
○------ Duration of filtration in the case of macerating with Na_2SO_3
▲------ Specific viscosity in the case of macerating with Na_2CO_3
●------ Specific viscosity in the case of macerating with Na_2SO_3

As shown in the table and the figure, the filtration of measured liquor obtained by macerating with sodium sulfite is remarkable easier than that of the liquor obtained by macerating with sodium carbonate. The fact may be explained as follows: reaction proceeds the material being always acidic in the case of maceration with sodium sulfite while it proceeds in alkaline state in the case of maceration with sodium carbonate.

The fluctuation of the data is due to the circumstance that the experiments were carried through by two assistants who worked alternatively on every maceration; the maceration process is furthermore affected by the condition of stirring, treating temperature and other unaviodable factors and constant results would not be expected even if close attention were paid in each treatment. Nevertheless, a remarkable difference of filterability is observed, on the whole, between the two macerating procedures as described above.

Viscosity of filtrate:

The filtrability of the macerated liquor would be affected by the viscosity of the filtrate. Specific viscosity of the liquor resulting from maceration with sodium sulfite is lower than that from sodium carbonate. The particularly low viscosity in the case of maceration with 0.5 g of Na_2CO_3 or 1.0–2.0 g of Na_2SO_3 is dependent upon imperfect maceration as is clearly shown in the yield of alginic acid. Remarkable ease of filtration is observed when the algae are pretreated with dilute hydrochloric acid and macerated with Na_2SO_3.

Yield of alginic acid:

The yield of alginic acid is higher in the case of maceration with sodium carbonate solution. The reason may be considered to lie in the circumstance that the cation exchange reaction between alginic acid and sodium carbonate would be perfectly irreversible because the reaction is a neutralization, while the cation exchange between alginic acid and sodium sulfite would be partly reversible because sulfurous acid is a stronger acid than alginic acid. It is recommended to use sodium sulfite for maceration of algae after the pretreatment by dilute hydrochloric acid solution in view of the ease of filtration, though it somewhat reduces the yield of algin.

Viscosity of the product:

The viscosity of thus obtained algin products was compared as follows: the preparations of alginic acid by both methods were neutralized with sodium carbonate solution and specific viscosity of thus obtained sodium alginate solution was measured. The results are shown in Table 23.

Table 23

Concentration of sodium alginate (%)	Macerated with Na_2CO_3 1.5 g	Macerated with Na_2SO_3 3.0 g
0.500	100.3	112.0
0.400	59.6	65.1
0.300	39.9	45.3
0.250	25.2	31.0
0.150	13.5	15.8
0.075	7.1	9.0
0.040	3.2	4.2
0.025	2.0	2.2

It is proved that the viscosity of algin preparation obtained by macerating with sodium sulfite is rather higher than that of the preparation obtained by macerating with sodium carbonate although the viscosity of filtrate macerated with sodium sulfite is remarkably lower than that of filtrate macerated with sodium carbonate.

Generally speaking, it would be a recommendable procedure for algin manufacture to pretreat algae at first with dilute hydrochloric acid solution in order to convert algin in the algae into free alginic acid and then to macerate them with sodium sulfite solution.

3. Process of Filtration of Macerated Liquor

The filtration of macerated liquor is one of the most difficult processes in algin manufacture. In practice, it is usually done by filter bag, filter press or centrifuge. Some investigations on the filtration were carried out with filter bags as described in the following.

The filter bags used for this study were made of cotton; they measured 17 cm in diameter and 245 cm in length. Dried *Laminaria* fronds were pretreated with 0.1 n hydrochloric acid solution added to the amount of 15 times the weight of algal fronds, at 50°C for 30 minutes and macerated with as much sodium carbonate as 15 per cent by weight of the fronds at 60°C for an hour. The macerated mass was diluted with water to 7 times by volume and poured into the bags. The experiments were made in August in order to learn the effect of decomposition by microorganisms. The results are shown in Table 24; the figures are mean value of the data from 10 bags. The specific

Table 24

Duration of filtration (min)	Filtrate at each period (cc)	Filtrate in total (cc)	Specific viscosity of filtrate	Yield of alginic acid (%)	Alginic acid in filtrate at each period (g)	Alginic acid in filtrate in total (g)
30	3950	3950	54.8	0.24	9.45	9.45
60	1200	5150	44.8	0.17	2.25	11.70
90	600	5750	43.2	0.24	1.44	13.14
120	400	6150	30.4	0.33	1.32	14.46
150	350	6500	24.0	0.13	0.52	14.98
180	250	6750	20.8	0.18	0.45	15.43
210	215	6965	17.0	0.16	0.34	15.77
240	175	7140	16.6	0.17	0.30	16.07
270	150	7290	15.0	0.15	0.23	16.30
300	125	7415	14.2	0.14	0.18	16.48

viscosity of the filtrate and the yield of alginic acid from the filtrate per dl. were also determined. The results are illustrated in Fig. 60.

The amount of filtrate is distinctively decreased with the duration of the filtration as shown in the table and fiugre, over 80 per cent of the total filtrate is obtained within 120 minutes. Alginic acid is also obtained to as much as 80 per cent of its total within 90 minutes. The specific viscosity of filtrate is remarkably decreased by the duration

of the filtration, therefore it is clearly indicated that decomposition of algin does take place during the process of filtrating the macerated liquor.

It is considered from the above described results that the decomposition of algin would be caused to occur by the long spells of filtration, and therefore the filtration of macerated liquor should be done mechanically in order to avoid the decomposition of the algin in the filtrate.

It has been reported by the author[37)38)] that alginic acid is decomposed by natural fermentation into acetic acid, so the application of some antiseptic treatment to the filtrate is worth considering in order to prevent the decomposition of algin due to microorganisms. However the use of such antiseptic treatment should be avoided in a factory, where algin is used as a food stuff, for instance as the stabilizer of ice-cream.

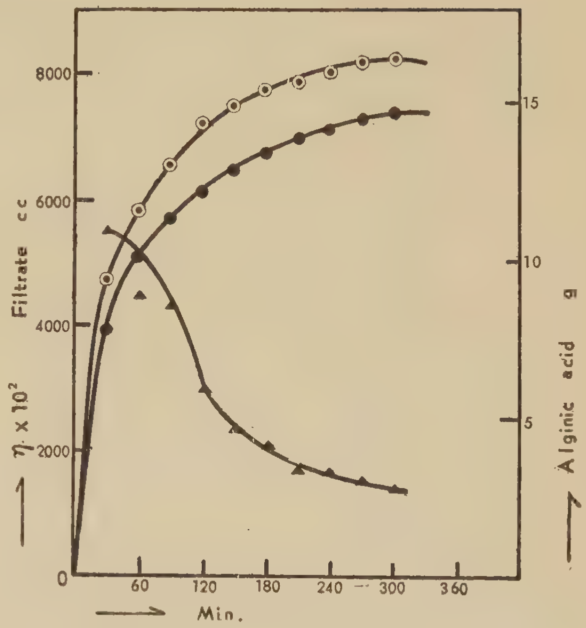


Fig. 60. Filtrate (cc), specific viscosity of filtrate and yield of alginic acid (g) in the filtrate in relation to the duration of filtration of macerated liquor

⊙------ Yield of alginic acid in total (g)
●------ Filtrate in total (cc)
▲------ Specific viscosity

4. Gelling of Alginic Acid

Though it has been customary to get gel of alginic acid from the filtrate by adding hydrocholoric acid solution, there has appeared no report regarding the formation of alginic acid gel by adding hydrochloric acid to the filtrate of macerated liquor. Some fundamental investigations on the conditions of gel formation were carried out as follows. To be used for these investigations alginic acid powder was prepared by the author's method and purified by ordinary method ; 0.3–0.7 per cent solutions of sodium alginate were prepared by neutralizing alginic acid with sodium hydroxide solution because gel of alginic acid is customarily made in the per cent of 0.3–0.7.

(i) *Experimental*

Ten cc of sodium alginate solution were pipetted into a test tube, to which was added 10 cc of hydrochloric acid solution of various concentrations. Then the mixture was shaken well. Produced alginic acid gel was collected on the filter paper under

Table 25. Yield of alginic acid (%) gelling with hydrochloric acid

HCl in the mixed solution (n)	Concentration of sodium alginate (%)				
	0.3	0.4	0.5	0.6	0.7
2.0	90.2	96.4	96.3	96.4	85.3
1.75	93.5	95.2	96.7	93.0	79.0
1.5	97.1	96.0	92.3	86.1	70.1
1.25	—	95.0	90.0	—	—
1.0	97.6	93.0	85.0	72.3	67.8
0.75	91.3	95.3	78.6	68.0	57.0
0.5	94.5	89.5	76.0	61.0	53.5
0.25	78.3	68.0	57.1	40.0	36.0
0.125	60.7	46.5	37.3	32.0	20.0
0.063	39.4	27.1	15.8	10.5	—
0.030	21.3	9.0	—	—	—

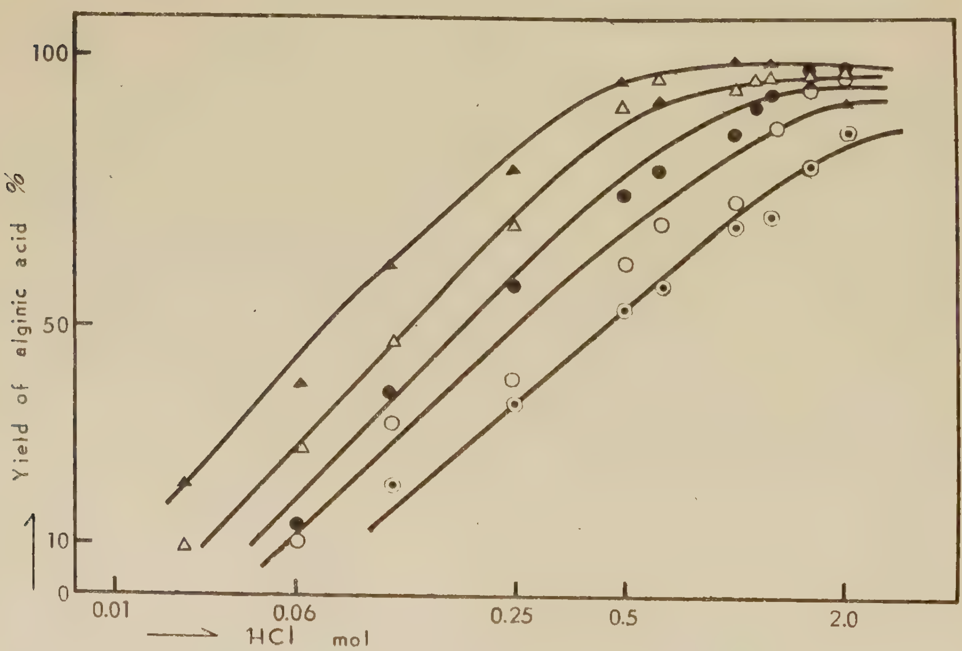


Fig. 61. Yield of alginic acid by gelling with HCl from sodium alginate solution

▲------ Gelled from 0.3% sodium alginate solution
△------ Gelled from 0.4% sodium alginate solution
●------ Gelled from 0.5% sodium alginate solution
○------ Gelled from 0.6% sodium alginate solution
⊙------ Gelled from 0.7% sodium alginate solution

reduced pressure and washed with hot water and then with ethanol and ether. Thus obtained alginic acid gel was dried at 95°C and weighed.

(ii) *Results*

The results are shown in Table 25 and Fig. 61.

It is shown that more hydrochloric acid solution is needed for getting gel from fairly concentrated sodium alginate solution.

The data shown in Table 25 and Fig. 61 are calculated in Tables 26–30 and Fig. 62 as to give the equivalent ratio of added hydrochloric acid for dissolved sodium alginate and to give the yield of alginic acid recovered by gelling with hydrochloric acid solution.

Table 26. Yield of alginic acid (%) by gelling with HCl from 0.3% sodium alginate solution

Added HCl (n)	Added HCl (mol) to dissolved Na-alginate (mol)	Yield of alginic acid (%)
4.0	248	90.2
3.5	217	93.5
3.0	196	97.1
2.0	124	97.6
1.5	93	91.3
1.0	62	94.5
0.5	31	78.3
0.25	15.5	60.7
0.125	7.8	39.4
0.063	3.9	21.3

Table 27. Yield of alginic acid (%) by gelling with HCl from 0.4% sodium alginate solution

Added HCl (n)	Added HCl (mol) to dissolved Na-alginate (mol)	Yield of alginic acid (%)
4.0	186	96.4
3.5	163	95.2
3.0	134	96.0
2.5	116	95.0
2.0	93	93.0
1.5	67	95.3
1.0	46.5	89.5
0.5	23.8	68.0
0.25	11.6	46.5
0.125	5.8	27.1
0.063	2.9	9.0

Table 28. Yield of alginic acid (%) by gelling with HCl from 0.5% sodium alginate solution

Added HCl (n)	Added HCl (mol) to dissolved Na-alginate (mol)	Yield of alginic acid (%)
4.0	149	96.3
3.5	130	96.7
3.0	112	92.3
2.5	93	90.0
2.0	74.4	85.0
1.5	55.8	78.6
1.0	37.2	76.0
0.5	18.6	57.1
0.25	9.3	37.3
0.125	4.7	15.8

Table 29. Yield of alginic acid (%) by gelling with HCl from 0.6% sodium alginate solution

Added HCl (n)	Added HCl (mol) to dissolved Na-alginate (mol)	Yield of alginic acid (%)
4.0	124	96.4
3.5	109	93.0
3.0	93	86.1
2.5	78	—
2.0	62	72.3
1.5	46.5	68.0
1.0	31	61.0
0.5	15.5	40.0
0.25	7.8	32.0
0.125	3.9	10.5

Table 30. Yield of alginic acid (%) by gelling with HCl from 0.7% sodium alginate solution

Added HCl (n)	Added HCl (mol) to dissolved Na-alginate (mol)	Yield of alginic acid (%)
4.0	106	85.3
3.5	93	79.0
3.0	80	70.1
2.5	66.5	—
2.0	53.2	67.8
1.5	40	57.0
1.0	26.6	53.5
0.5	13.3	36.0
0.25	6.7	20.0

(iii) *Discussion*

From the above data is clearly learned the fact that an enormous quantity of hydrochloric acid is needed in order to make gel of alginic acid from sodium alginate solution. In a comparatively higher concentrated solution of sodium alginate, the equivalent ratio of hydrochloric acid would need to be still large. That is to say, about 60 mol of hydrochloric acid is required for 1 mol of alginate in 0.3 per cent sodium alginate solution, but over 100 mol of hydrochloric acid is required in 0.7 per cent sodium alginate solution in order to recover 90 per cent of algin in the solution. Moreover, a considerable amount of hydrochloric acid is consumed in a factory for neutralizing alkali which was used to macerated algae, so that requirement of hydrochloric acid amounts to a very large quantity in algin industry.

5. Drying of Algin

Alginic acid obtained by the above described methods should be washed and dehydrated. However, the dehydration of swelling water in alginic acid gel is very difficult by washing with water. The author proposes to wash the gel with hot water at 55–58°C, in accordance with what he was learned by actual experience. Such a

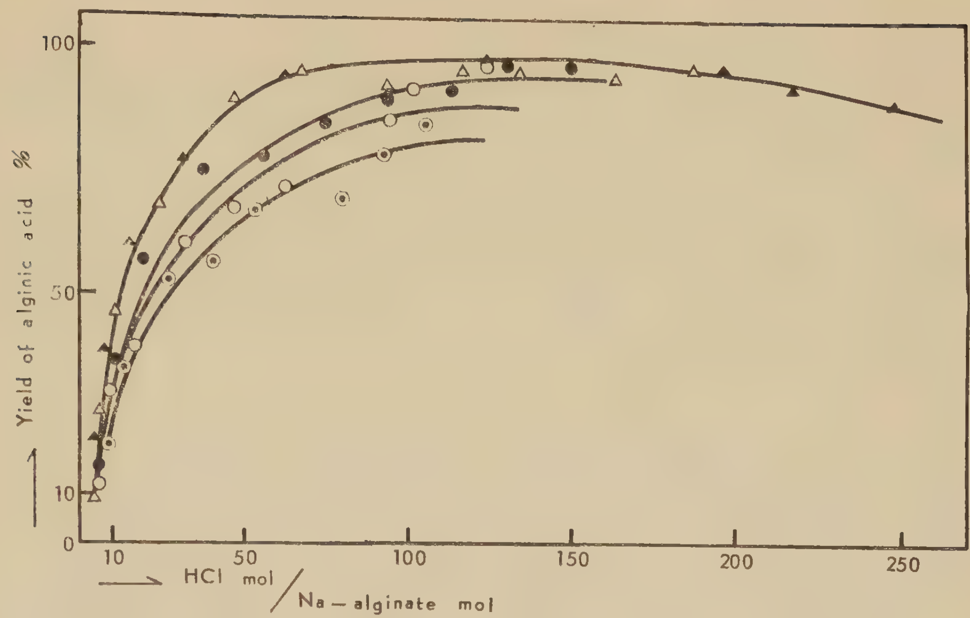


Fig. 62. Yield of alginic acid and equivalent ratio of HCl to sodium alginate (HCl mol/Na-alginate mol) when alginic acid is gelled with HCl from sodium alginate solution

▲------ Gelled from 0.3% sodium alginate solution
△------ Gelled from 0.4% sodium alginate solution
●------ Gelled from 0.5% sodium alginate solution
○------ Gelled from 0.6% sodium alginate solution
⊙------ Gelled from 0.7% sodium alginate solution

mild heating would make the dehydration of the gel very easy and at the same time would not decrease its viscosity. Further dehydration of thus obtained alginic acid gel can easily be done by centrifuging or pressing. The contracted gel thus obtained contains ordinarily about 80–90 per cent water, while alginic acid gel obtained without heating contains swelling water 200 times as much as its weight.

Algin is marketed in various types of combination prepared by various drying procedures. Sodium alginate and alginic acid are the commonest products of algin which are on the market. The drying process in the preparation of these two products of algin were investigated in the present study.

There are two ways for producing sodium alginate in a factory. One is by drying sodium alginate paste itself by some drying method, and the other is by preparing at first sodium alginate gel in 30–40 per cent ethanol solution by neutralization of alginic acid gel with sodium carbonate or caustic soda and drying thus obtained solid sodium alginate gel by some drying process. However, both methods are accompanied by a process of drying sodium alginate. The effects on viscosity of alginate by heating sodium alginate paste are investigated in connection with the preparation of sodium alginate.

(i) *Drying of sodium alginate*

Sodium alginate preparation was made from sodium alginate solution by repeating dissolution with water and gelling with ethanol several times. Then the gel was washed with ether and dried in desiccator.

Twenty five cc of 1 per cent sodium alginate was pipetted into each of number of petri-dishes and heated at 70, 80, 90 and 100°C respectively. After heating for a certain period 0.25 per cent solution of sodium alginate was prepared and its specific viscosity was measured. The relative values of the viscosity were calculated adjusting initial viscosity of the solution to 10.00, where observed initial viscosity of the solution was 90.00. The results are shown in Table 31.

Table 31. Viscosity of 0.25% Na-alginate solution

Duration of heating (min)	Temperature of drying (°C)			
	100	90	80	70
0	10.00	10.00	10.00	10.00
30	7.23	7.15	9.10	9.28
60	4.55	5.97	8.69	—
90	3.23	4.39	7.60	8.80
120	2.69	4.50	7.66	—
150	2.77	3.92	7.07	—
180	—	—	—	8.22

It is considered that the colloidal quality of sodium alginate would not be affected very markedly by heating at 70°C, however, the effect of heat upon dried film of sodium alginate should be examined because the heat attack upon dried film of sodium alginate does not occur under such a condition.

Undesirable effect of heat upon the viscosity at lower temperature in concentrated solution of sodium alginate was investigated as follows. Twenty grams of 2.5 per cent sodium alginate was weighed in a petri-dish, the change of specific viscosity and the weight of the paste were determined and calculated to initial values 10.00 ; the results are shown in Table 32 and Figs. 63 and 64.

Table 32

Duration of heating (min)	Temperature of drying (°C)						
	40		50		60		70
	Viscosity	Weight	Viscosity	Weight	Viscosity	Weight	Weight
0	10.00	10.00	10.00	10.00	10.00	10.00	10.00
30	10.20	9.33	10.00	8.31	8.35	6.95	5.87
60	10.67	9.08	9.70	7.85	8.04	4.28	4.05
90	11.15	9.02	9.10	7.23	—	3.25	2.55
120	14.13	8.59	5.58	6.85	—	1.87	1.38
150	14.75	8.18	5.13	5.89	—	—	—
180	17.05	7.74	3.78	—	—	0.75	0.38

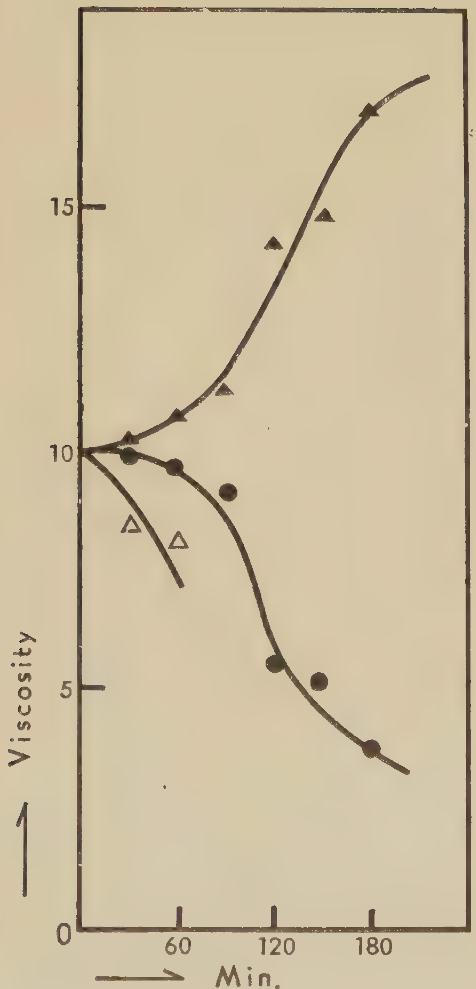


Fig. 63. Change of specific viscosity of 2.5% sodium alginate solution by heating

△------ Heated at 40°C
●------ Heated at 50°C
△------ Heated at 60°C

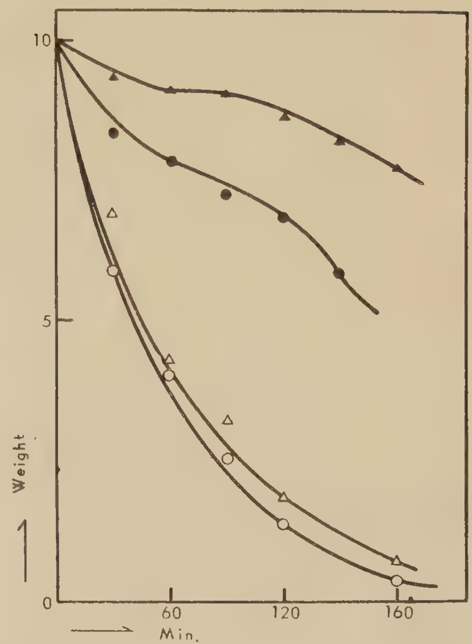


Fig. 64. Reducing of the weight of 2.5% sodium alginate solution by heating

▲------ Heated at 40°C
●------ Heated at 50°C
△------ Heated at 60°C
○------ Heated at 70°C

The decrease of viscosity caused by heat attack is observed more distinctively as is shown in the table and in Fig. 63 than in the case of heating of dilute solution, though the viscosity of preparations treated at 40°C is increased by heating. The viscosity of concentrated sodium alginate solution is often observed to increase at lower temperatures, sometimes even at lower than 40°C. The reson of this increase is not clearly known yet.

As it is suggested that the colloidal quality of sodium alginate is attacked especially by heating after it is dried in the form of film. The heat attack to solid sodium

alginate was investigated as follows.

Solid sodium alginate, 1.00 g was heated at 40–70°C for 30–150 minutes and specific viscosity of each treated preparation was determined. The measured values are shown in Table 33 and Fig. 65 adjusting to initial value 10.00.

Table 33. Change of viscosity by heating to solid sodium alginate

Duration of heating (min)	Temperature of heating (°C)			
	40	50	60	70
0	10.00	10.00	10.00	10.00
30	9.90	10.00	9.25	7.10
60	9.70	9.40	8.85	6.22
90	9.15	8.80	8.52	5.47
120	9.07	8.22	7.77	5.15
150	9.07	7.37	7.15	5.05

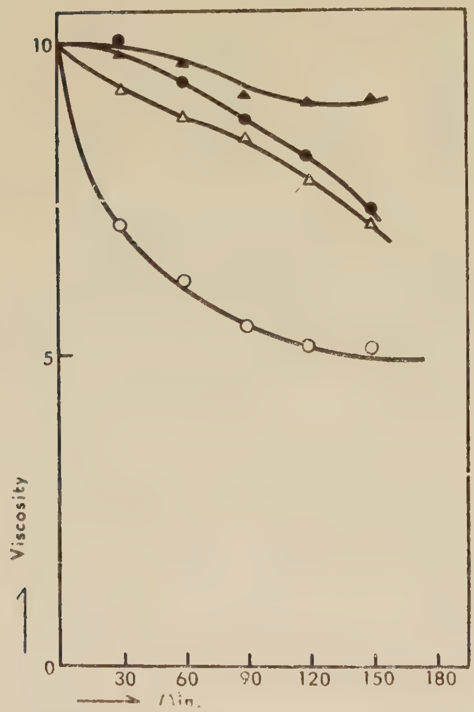


Fig. 65. Change of viscosity of solid sodium alginate by heating

▲------ Heated at 40°C
●------ Heated at 50°C
△------ Heated at 60°C
○------ Heated at 70°C

Exposure to heat over 50°C is proved to cause the decrease of the viscosity of solid sodium alginate.

(ii) *Drying of alginic acid*

The alginic acid gel is swollen by water very largely. Actually, the swelling water amounts 200 times the weight of the original gel substance. Consequently the dehydration of the gel is very difficult as described above. However, the author has succeeded to dehydrate the swelling water of alginic acid gel, after many experiments by means of heating the gel in water at 55–58°C as described above. The alginic acid gel thus dehydrated, containing 82.15 per cent water, was used for the investigations of drying. Each 5.0 g portion of alginic acid gel was placed on petri-dish and dried at 40, 50, 60, 70 and 80°C respectively for a certain period ranging from 30 to 180 minutes. The changes of viscosity were measured on 0.25 per cent sodium alginate solution prepared from heated alginic acid gel. The results are shown in Tables 34 and 35 and Figs. 66 and 67.

Table 34. Change of weight of alginic acid gel by drying

Duration of heating (min)	Temperature of heating (°C)				
	40	50	60	70	80
0	10.00	10.00	10.00	10.00	10.00
30	8.05	5.78	5.35	4.82	2.45
60	6.17	2.90	2.85	2.83	1.27
90	5.88	2.40	1.73	1.34	1.24
120	4.80	2.02	1.71	1.27	1.24
150	4.08	1.73	1.34	1.27	1.24
180	3.95	1.42	1.27	1.27	1.27

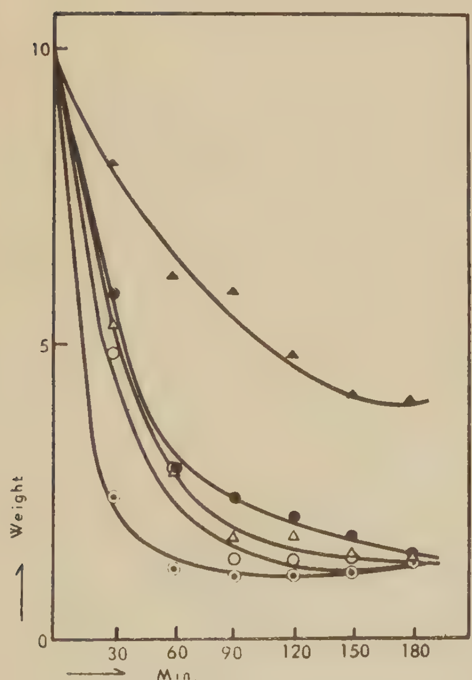


Fig. 66. Change of the weight of alginic acid gel by drying

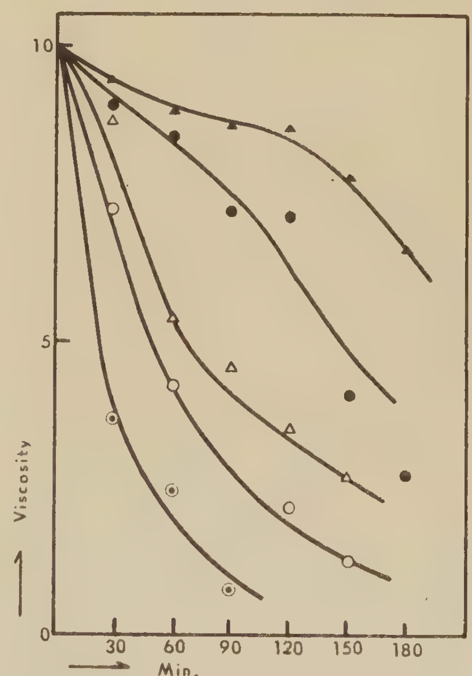


Fig. 67. Change of 0.25% sodium alginate by drying

▲------ Heated at 40°C ●------ Heated at 50°C △-----Heated at 60°C
○------ Heated at 70°C ⊙------ Heated at 80°C

It is clearly that the viscosity of alginic acid is remarkably decreased by heating at higher temperature and heating the dried surface of alginic acid gel. In order to prevent this effect of heating, alginic acid should be shredded into small pieces

Table 35. Change of viscosity of alginic acid gel by drying

Duration of heating (min)	Temperature of heating (°C)				
	40	50	60	70	80
0	10.00	10.00	10.00	10.00	10.00
30	9.40	8.95	8.75	7.30	3.67
60	8.90	8.42	5.38	4.23	2.43
90	8.65	7.14	4.57	3.14	0.79
120	8.57	7.14	3.49	2.15	0.67
150	7.76	4.08	2.65	1.25	—
180	6.50	2.71	—	—	—

uniformly and dried at temperatures lower than 50°C in presence of relatively wet air aiming to prevent the heat attack upon the dried surface of alginic acid gel.

CONCLUSION

Since there have been published only a few works on the theories involved in the mechanism of manufacturing algin from brown algae, the author made these investigations.

Through microchemical examinations it was proved that algin exists in various types of combination. The existence of calcium alginate, even partly or mostly, is supposed in the cell wall of the *Laminaria* fronds. The algin which would be converted into calcium alginate when it is treated with calcium chloride solution seemed to exist in the cell wall of the brown algae. The existing state of algin in brown algae is considered to be not so simple but very complicated, therefore studies on the mechanism of algin manufacture would be impossible without converting algin into a certain definite combined form.

The author analysed a number of samples of brown algae which would be used as raw materials in algin manufacture and investigated seasonal, local and specific differences of their components. Since mannit, potassium or iodine would be obtained as by-products of algin industry, their contents were also examined. The obtained remarkable results are briefly described as follows.

Because the seasonal variations of the chemical components in the material effect each other in respect to their contents, the absoulte amount of each component in the material can not be discussed, but their relative contents can be. Very obvious seasonal variations are observed in the amounts of iodine, mannit, protein and fat. The former two components show their minimum amounts in spring and evidently increase toward autumn. On the contrary, the latter two show their maxima in spring and minima in autumn. This relationship is considered to be significant in the life of brown algae, because the photosynthesis of plants is at its maximum in summer and the metabolic processes are at their minima in winter. The contents of ash, potassium and alginic acid vary seasonally to some degree, but the variations could not be

distinguished from those which may be caused by alternative relation of other obviously varying components.

Alginic acid, from the above described consideration, would be considered a standing component of brown algae because it does not vary much with season or species. Alginic acid is considered to exist as an intercellular substance in the cell wall of brown algae, to play some important role in their skeleton. As for the role of algin in brown algae, further studies should be undertaken.

The brown algae should be harvested in autumn, at their reproductive period, because their growth reaches its maximum and the mannit and iodine contents show their maxima in that season, although algin contents do not vary so remarkably by season.

Algin, mannit, iodine and potassium are generally contained in larger amount in Laminariaceae than in Fucaceae. Therefore, it may be said that most suitable raw materials for algin manufacture and its by-products are the algae belonging to the Laminariaceae, though it should be taken into consideration in a factory that the Laminariceous algae are rather expensive.

Theoretical and practical studies on algin manufacture were carried out by converting algin in brown algae into free alginic acid and calcium alginate, pretreating algae with dilute hydrochloric acid or calcium chloride solution. Algin in brown algae exists in various types of combination as above described, so it is desirable to convert algin in brown algae into some definite combined form in order to manufacture algin by means of most suitable manufacturing process.

For the purpose of learinng the actual mechanism of manufacturing processes, fundamental investigations were made using purified free alginic acid and calcium alginate, and several properties of both kinds of algin are also studied as described next.

Alginic acid, from the results of determination of dissociation degree, pH, ionization constant and pK of the saturated alginic acid solution, is proved a fairly weak acid.

The titration curve of saturated alginic acid solution with sodium hydroxide is very characteristic and the reason of the fact is considered to be the existence of carboxyl radicals having various dissociation constants or some certain colloidal effect. The characteristic of titration curve does not disappear by adding electrolyte to alginic acid solution, therefore, the existence of carboxyl radicals having different dissociations is considered to be fairly possible although further investigations are needed because the colloidal effect would be eliminated by adding a fairly large amount of electrolyte to such a dilute colloidal solution.

Calcium alginate preparations were made by adding various amounts of 5 per cent calcium acetate solution to sodium alginate solution. Preparates were dialyzed in distilled water until Ca ion reaction in dialyzing water had disappeared. Since any

remarkable difference was not recognized concerning their solubilities and other properties in the range of calcium content in calcium alginate, 9.7 to 10.5 per cent, the preparates containing calcium in the range of 9.8 to 10.5 per cent were used for learning the mechanism of algin manufacture from calcium alginate, where the theoretical calcium content in calcium alginate calculated from the formula $C_6H_7O_6.1/2Ca$ is 10.25 per cent.

It has been made clear by studies reported in this paper that insoluble algin such as free alginic acid or calcium alginate, is dissolved into soluble alginate by cation exchange. The cation exchange equilibrium between insoluble algin and an electrolyte concerned with the dissolution of algin is presented in the following formula ;

$$\text{Alg-COOX}+\text{MA}\rightleftarrows\text{Alg-COOM}+\text{XA}$$

where, "X" represents a cation which forms an insoluble algin combining with Alg-COO^-, such as H or Ca, and "M" represents a cation which forms a soluble algin combining with Alg-COO^-, such as an alkali metal, ammonium radical or magnesium, and "A" represents an anion.

An insoluble algin is dissolved into soluble algin when the above mentioned reaction is almost or completely irreversible. That is to say, the dissolution of algin takes place in the special case of cation exchange reaction. The electrolytes which are able to be used for dissolving free alginic acid and calcium alginate are described respectively in Tables 7 and 4.

Since algin occurs in various states of combination in brown algae as described above, it is desirable to pretreat algae with a certain proper reagent solution in order to convert algin in brown algae into a definite form, because algin would be extracted by suitable manufacturing process according to the pretreatment. The author discovered that many sorts of electrolytes are available to extract algin from brown algae as described above. Among them two recommendable processes are described as follows.

1) *Algin manufacture from algae macerated with sodium sulfite*

It is observed that there are two cases in dissolution of free alginic acid into soluble alginate with the electrolyte in Table 7. One is the case where reaction proceeds with the material keeping acid, and the other is the case where the reaction proceeds with the material keeping alkaline. The reason is obvious that the reaction (2) proceeds keeping acid when "A" is an acid radical, while the reaction proceeds keeping alkaline when "A" is OH^- or CO_3^{--}. This is true since the latter is a case of dissolution by neutralization, and it may be said a peculiar case of cation exchange reaction which is perfectively irreversible.

Among the electrolytes enumerated in Table 7, Na_2SO_3 is a very highly recommendable one, because the macerated mass or diluted solution is protected from decomposition by microorganisms and algin is bleached by SO_2 which will be generated in the process of the reaction :

$$2Alg\text{-}COOH + Na_2SO_3 = 2Na\text{-}Alginate + H_2O + SO_2$$

Furthermore, the reaction is almost perfectly irreversible because the produced SO_2 would escape from the system by heating.

A practically manufacturing method of algin from brown algae with sodium sulfite is briefly described as follows.

In order to convert various types of algin in brown algae into free alginic acid, the algae are treated with 0.1 n hydrochloric acid solution for 15–30 minutes at 50–60°C. After removal of acid solution, 15 per cent of sodium sulfite by weight to dried algae and 30 times that weight of water are added to the remaining algae and heated for an hour at 60–70°C, then the bodies of the plants are completely disintegrated. Further treatment may be done as in an ordinary method.

2) *Algin manufacture from algae macerated with sodium chloride and sodium carbonate* .... (SUZUKI's *method*)

It is proved that algin can be manufactured by following procedure without destroying epidermal tissue and hence without dissolving out the pigment from the assimilatory cells; the author proposes to name this method "SUZUKI's method." The method is established by the fundamental investigation which is shown diagrammatically in Fig. 18, the method is developed as follows.

Treat the dried brown algae first with boiling water for 15–30 minutes to swell the plant body and to remove soluble matter and other impurities. Drain the water, and treat the algae with 0.5 per cent calcium chloride or saturated calcium hydroxide solution for 15–30 minutes at 80–100°C to convert algin in the cell walls into calcium alginate and to make the pigment in the epidermal cells insoluble. Cast away the liquor, and treat the algae with 10 per cent sodium chloride by weight to dried algal materials and 30 times that weight of water for 30 minutes at 80–100°C. Within this period, calcium alginate in the cell walls is swollen by cation exchange reaction producing soluble algin, while the pigment is retained in the plant body. Do not cast away the liquor, macerate the remaining bodies of algae with sodium carbonate added to as large amount as 10 per cent by weight of the original dried algae for 10–20 minutes with gentle stirring. Allow to stand for several hours so as to bring about complete maceration; the pigment and the epidermal cells would remain undissolved and undamaged. The colour of the algin thus obtained is almost white without any bleaching and its viscosity is very high owing to the weak attack of alkaline solution. The process of filtration is very easily carried out because epidermis and mucilagenous substances excepting algin are very slightly dissolved.

Further investigation regarding each treatment process in the manufacture of algin and its by-products is made as follows.

The pretreatment of brown algae should be done for two purposes, namely, first for the purpose of extracting mineral salts, mannit or soluble impurities and second

for the purpose of converting algin in brown algae into definite state of combination. Since only a few works are available for pretreatment of brown algae, several investigations were carried out in the present study. The extracting power of the pretreating solution is greatly affected by its effect in causing the algae to swell. It is proved that algae absorb water as much as about 3–4 times their weight and they absorb 1 per cent calcium chloride solution as much as 2.5–3.5 times their weight within a short duration of the treatment, while wet algae do not absorb water as much as 50 per cent of their weight. Dilute hydrochloric acid is absorbed only as much as 50 per cent of the weight of algae even the dried material. The extractive power of several solutions, such as water, 0.1 n hydrochloric acid, 1 per cent calcium chloride, 0.005 per cent and 0.001 per cent NaOH, 0.01 per cent Na_2CO_3, sea water and the mixture of fresh water and sea water was investigated. No remarkable difference of power to extract potassium was shown amongst the treatments. Some difference of power to extract iodine was observed amongst the treatments; the minimum power to extract iodine was observed in the case of pretreating with dilute hydrochloric acid solution and the maximum is shown in the case of alkali treatment. The yield of mannit showed fairly great fluctuation and it is suggested that mannit is less soluble than potassium chloride or iodine under the conditions above described. At all events, no essential difference in respect to extractive power could be found amongst the examined pretreatment, therefore, pretreatment of algae may be done only considerating the conversion of type of algin, though the extractive power as having effect upon pigment and other impurities should be taken into consideration. It is considered that high concentrations would be required in spite of lowering the yield by repeating pretreatments because the recovery of the matter is generally difficult and expensive when the solution is dilute.

Some fundamental investigations were made on the recovery of mannit by fractional crystallization from other soluble mineral matter.

Since only a few works are available on the processes of manufacturing algin from brown algae, the author investigated each process of manufacturing stages as follows concerning with the new methods which have been perfected by him.

As algin is fairly unstable in its colloidal qualities, close attention must be paid to every step throughout the process of manufacturing algin.

The viscosity of the soluble alginate is decreased by heating according to the following formula,

$$y = Ae^{-\alpha t}$$

where, y represents specific viscosity of each stage of the duration of heating, t represents duration of heating and α and A are the constants of the equation. It is clear that the viscosity of algin would be decreased by heating, therefore, long spells of heating and treatment with high temperature should be avoided on every step in the

process of manufacturing algin.

The macerating processes with several chemicals were investigated pretreating algae with three solutions, water, dilute hydrochloric acid and dilute calcium chloride solution. The maceration was promoted by using sodium carbonate, sodium hydroxide, sodium sulfite, sodium dibasic phosphate and ammonium oxalate; the yield of alginic aicd, specific viscosity of the preparations and the colour tone of alginic acid were examined. From the results, it is proved that sodium carbonate, sodium sulfite and sodium dibasic phosphate are able to macerate algae with every one of the used pretreatments, sodium hydroxide can macerate algae with dilute hydrochloric acid pretreatment only and, on the contrary, ammonium oxalate can not macerate algae with hydrochloric acid pretreatment. Those facts may be explained by the fact that the cation exchange reaction between sodium hydroxide and free alginic acid is irreversible but the cation exchange reaction between sodium hydroxide and calcium alginate or other insoluble alginate in the brown algae is almost reversible, and free alginic acid is not dissolved into colloidal solution by treating with ammonium oxalate solution although it is dispersed into small particles as described in Table 3. For this reason it is that algin is scarcely dissoluble into the solution from the cell wall of the algae. From the examination of the yield, viscosity and colour tone of the algin preparations, it may be said that the most excellent way of algin manufacture among the experiments is to macerate algae with sodium sulfite after pretreating with dilute hydrochloric acid solution.

Filtrability of the macerated liquor was studied after maceration with above mentioned chemicals in the case of pretreating with water and dilute hydrochloric acid solution. It is also proved that maceration with sodium sulfite after pretreatment with dilute hydrochloric acid solution is the most excellent.

From the examination concerning the filtration of macerated liquor it is suggested that decomposition of algin would be caused by long spells of filtration and, therefore, the filtration of macerated liquor should be done mechanically in order to avoid the decomposition caused by long spells of filtration.

It is clear that an enormous amount of hydrochloric acid for alginic acid is needed to gel alginic acid from sodium alginate solution. That is to say, about 60 mol of hydrochloric acid is needed for gelling 1 mol of alginic acid from 0.3 per cent sodium alginate solution and over 100 mol of hydrochloric acid is needed for gelling 1 mol of alginic acid from 0.7 per cent sodium alginate solution. Moreover, in a factory, hydrochloric acid is expended for neutralizing alkali which is used for macerating algae, therefore, the maceration agent must be used as economically as possible in algin industry.

The viscosity of algin is remarkably decreased by heating, especially decreased by heating the dried surface of solid algin. Therefore, algin should be dried with careful management in order to prevent the above mentioned effect.

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STUDIES ON THE TROPOMYOSIN OF SQUID

KATSUJI YOSHIMURA
Faculty of Fisheries, Hokkaido University, Hakodate, Japan

CONTENTS

I. INTRODUCTION

The squid (*Ommastrephes sloani pacificus* STEENSTRUP) belongs to the cephalopoda mollusca. The squid muscle is soft and smooth and systematically, chemically and physicochemically is very peculiar as compared with those of the animals and fishes. The kinds and the amounts of amino acid which build up the muscle protein have been estimated by many persons. The author[1] also had determined the quantitative and qualitative differences of amino acids of squid muscle protein and extract and had found remarkable differences between squid and fishes.

Recently MIGITA[2] has studied on the squid muscle and found the differences of various properties between squid muscle and fish muscle and submitted some consideration on the origin of these differences.

The first difference is that the squid muscle can be readily torn off laterally in fibers, for the squid muscle fibers come together in bunches and are arranged in parallel.

The second is that the squid muscle shows marked contractiveness and the contractile character differs with direction. These facts are caused also by the fact that the muscle fibers are arranged in parallel and that contractile character differs with direction.

The third difference is that it is far more readily soluble in water than fish meat. Such a difference might be caused by the physicochemical and chemical properties of protein molecules and also by the existence of various substances other than protein

which are included in the muscle.

The fourth difference is that the squid muscle shows such a strong swelling ability in a salt solution as often to lead to peptisation under the condition in which fish meat does not exceed the swollen state, and such a strong swelling ability is caused by the excellent hydrochemical properties of squid muscle.

The fifth difference is that the isoelectric point of squid muscle protein measured from the minimum swelling or the maximum precipitation point, is lower than that of fish muscle protein. It is caused by the fact that the squid muscle protein includes a larger amount of acidic amino acids and less amount of basic amino acids than the fish muscle protein.

The sixth difference is that an aqueous extract from squid meat even of the highest freshness gives precipitation reaction with mercuric chloride, which is positive in fish meat, as a rule, only after spoilage has begun.

Seventh difference is that the squid muscle shows intense streaming birefringence not only in salt solution but also in aqueous extract. The salt solution of squid muscle and aqueous extract shows intense streaming birefringence. Ordinarily such a streaming birefringence appears only in neutral salt solution of fish muscle other than squid, but in the case of squid such a streaming birefringence is kept a long time at a low temperature. The cause of such a phenomena is due to the fibrous structure of such a protein molecule as myosin.

The aqueous extract of squid muscle included myosin and it remained still in the fibrous structure even after slight autolysis.

Various such differences between squid and other fish muscle were caused by the specific properties of protein and the various substances other than proteins in the squid muscle.

KITABAYASHI[3] has estimated the amount of myosin, myoalbumin, myogen and globulin-x of the squid muscle by the method of SNOW *et al.*,[4] the results are shown in Table 1.

Tabl 1. Protein-composition of squid muscle

Kinds of protein	Squid*	Cod**
Myosin	9	65
Myoalbumin	54	1
Myogen	24	10
Globulin-X	4	19
Stroma	5	5

* KITABAYASHI[3] ** SNOW *et al.*[4]

From the above table it is seen that the amount of squid muscle proteins differs much from that of fish, that is, the amount of myosin of cod's muscle is about 7 times as large as that of squid. More than a half part of the protein of squid muscle is myoalbumin but in the cod the amount of myoalbumin is only a trace.

The actin component of STRAUB,[5] studied in some detail by SZENT-GYÖRGYI[6] and the SZEGED school, must also be considered as part of the fibril. Indirect assay suggests that the proportions of myosin and actin are in the ratio 8 : 3 (BALENOVIĆ &

STRAUB[7]). Actin possesses the property of converting myosin sols into a thixotropic gel, an interaction which involves the sulphydryl groups of the myosin partner.[8][9]

KITABAYASHI[10] has estimated the amount of myosin, actin, and tropomyosin in the squid muscle and flat fish muscle ; the proportions of mysoin and actin are in the ratio 1 : 1 for the fresh squid muscle and 5 : 1 for the fresh flat fish muscle. As mentioned above the amount of various kinds of protein in the squid muscle is much different from those of the fishes.

The myosin of the sarcoplasma is largely enzymic in function. ENGELHARDT & LJNBIMOVA[11] first showed that isolated myosin is associated with adenosine triphosphatase activity ; subsequent attempts to separate the enzyme from myosin have been unsuccessful.

When the adenosine triphosphate was added to the myosin solution it caused variations in viscosity,[12] streaming birefringence,[13] and scattering of light[14] of myosin solution, and the variation in the physicochemical properties of myosin molecules was observed. At the time when the adenosine triphosphate is catalytically hydrolysed by the adenosine triphosphatase (myosin) energy produced and it is used to contract the myosin itself.

The fact that actomyosin was the filamentous gel of myosin contracted by the adenosine triphosphate and inorganic salt, was first observed by SZENT-GYÖRGYI.[15]

The converting of muscle or the mutual relationship between adenosine triphosphate and myosin, is the conversion of energy in the living body from the chemical to mechanical, and many students have given attention to a clarification of the relation between these phenomena.

Studies of the myosin as an enzyme became the object of researchers' attention.

Tropomyosin is a new fibrillar protein of asymmetric character and relatively low molecular weight, quite distinct in all its major properties from actin.

Tropomyosin has been crystallized from rabbit muscle by BAILEY[16] in 1948. From its amino acid pattern,[16] and X-ray and electromicroscopic investigation,[17] it is found to be prototype of a much larger myosin molecule and might conceivably be a unit utilized in the elaboration of myosin itself.

Recently, detailed accounts of amino acid analysis[18] with Dowex-50 resin column and of the investigations of N- and C- terminal residues[19][20] have been published with rabbit tropomyosin. It has been observed that tropomyosin contains a large amounts of polar amino acid groups and is composed of a large cyclic polypeptide chain.

In the present investigation, tropomyosin of squid muscle was newly crystallized.

It was found not to be nucleotropomyosin judging from the results of examination of total phosphorous content and ultraviolet absorption ; remarkable differences were observed in amino acid composition, electrophoretic mobility and sedimentation constant, as a matter of course in crystallized condition.

From the squid muscle, which had remarkable differences in respect to the

systematical, chemical and physicochemical properties compared with other animal muscle, also could be separated the tropomyosin in pure crystalline state. From these facts the author considers it to be that the tropomyosin is a preform of myosin, and that the myosin had synthesized through the tropomyosin as previously stated by BAILEY.

To explain the chemical properties and physicochemical meaning of squid muscle myosin, it is very important to make studies of the physicochemical and chemical properties of squid tropomyosin.

The author wishes to express his heartiest thanks to Professor Yukihiko Nakamura, Faculty of Agriculture, Hokkaido University, for his invaluable advices and constant encouragement throughout the course of the present study. Cordial thanks are also due to Professor Yataro Obata, Faculty of Agriculture, for his kind criticism of the experiments in the present studies. He is much indebted to Mr. Shuichiro Kubo, Faculty of Fisheries, Hokkaido University, for generous assistance in carrying out the experiments.

II. EXPERIMENTAL RESULTS

1. Preparation and Crystallization

Preparation of tropomyosin : Fresh squid muscle is minced, mixed with an equal volume of water, and homogenized for 1 min. and squeezed out in muslins after addition of equal volume of ethanol. The washings are repeated twice with 97% ethanol and then twice with ether. The fibre is dried in air at room temperature and, while still ether-damp, is immersed in M-KCl (700 ml/100 g fibre) and the pH of the suspension is adjusted to 7 with N-NaOH. After standing for 12 hrs. at 15–20°C, the suspension is pressed through muslin. The residue is now re-extracted as before with M-KCl for 1 to 2 hrs. and the liquid is squeezed out in a hand press.

The pH of the combined extracts is adjusted to 4.3 with N-HCl and after 1 hr. the whole is filtered and precipitate is dissolved in 5 vol. of water, then the pH is readjusted to 7 with N-NaOH. The total volume is measured and 0.7 vol. of saturated ammonium sulphate (containing 0.01 vol. of conc. liquid ammonia) is added with stirring. The flocculum of denatured protein is filtrated, leaving a clear filtrate to which solid ammonium sulphate is added in portions to 70% saturation. The precipitate of tropomyosin is filtered off under gravity continued overnight, dissolved in water and dialyzed for 24 hrs. against a large volume of water.

The dialyzed solution is treated with HCl to the point of maximum precipitation (near pH 5.3), and the precipitate centrifuged, dispersed in water and neutralized to pH 7. The salting out procedure described above is repeated.

Crystallization : Precipitate of tropomyosin obtained by twice-repeated salting

out to 70% saturation is suspended in small amount of water and dialyzed against water of pH 5.8 at 10°C; it is then stored at 4°C for 2 days to increase white turbidity matter. Crude needle crystals are deposited on the cellophane membrane (Fig. 1) and collected by centrifugation. These crude crystals are dissolved in 0.2 M KCl of pH 7.2 and 2–3% protein solution then dialyzed at 0°C against 0.01 M phosphate buffer of pH 6.5 containing 12 g ammonium sulphate per liter. After standing 1 or 2 days needle crystals are deposited on cellophane membrane. Recrystallizations are repeated twice (Fig. 2).



Fig. 1. First crystals of tropomyosin × 1000



Fig. 2. Thrice crystals of tropomyosin × 400

Crystals are filtered on a sintered glass filter and dried through several changes of ethanol and ether, and stored in refrigerator. The squid tropomyosin is stable in organic solvents just as rabbit tropomyosin is.

The general properties of the tropomyosin are shown in Table 2.

Table 2. Composition of squid tropomyosin compared with rabbit and carp tropomyosin

	Total N	Tryptophane	Total phosphorus
Squid	16.68	0	0.07
Rabbit*	16.7	0	0.02
Carp**	16.7	—	0.20

* BAILEY[16)]　** HAMOIR[21)]

Tryptophane is not included in this protein; tryptophane content is an index of the purity of tropomyosin according to BAILEY.[16)] In this sample tryptophane is not estimated by means of the method of GOODWIN & MORTON,[22)] and of p-Dimethyl amino benzaldehyd.[23)] Differences according to animal kinds are not observed in nitrogen contents.

2. Assay of Tropomyosin

The minced muscle (100 g) is homogenized for 1 min. with an equal volume of water in a blender and squeezed out in muslin after addition of 200 ml ethanol. The residue is dried in ethanol and ether as before. It is then extracted with 100 ml M-KCl at pH 7, first for 12 hrs. at 15–20°C, with further change of KCl solution. The protein in the combined extracts is precipitated at pH 4.3 with N-HCl, centrifuged; the precipitate is dispersed in water (80 ml); the solution is neutralized to pH 7 and treated with saturated ammonium sulphate solution (containing 0.01 vol. conc. ammonia solution) to 41% saturation. The precipitate, after centrifuging, is triturated with 2 parts of 41% saturated ammonium sulphate solution, and again spun down. The combined supernatant fluid is treated with solid ammonium sulphate to 85% saturation, and the precipitated protein is filtered off under gravity. It is then dissolved in 100 ml water and dialyzed for 4 days against frequent changes of 0.1 M-KCl solution of pH 7.2. To a 10 ml sample of the dialyzed sol is added 0.2 ml M-phosphate buffer of pH 7.2, and the solution is heated in a boiling water bath for 10 min. After cooling for 1 hr. the slight precipitate of denatured protein is spun down, leaving tropomyosin in solution. The protein content is determined by estimating the total N and correcting for free NH_3 by distilling an undigested portion of solution at pH 9.5. The protein N content $\times$ 6 gives the yield of tropomyosin.

Tropomyosin content in squid muscle is 0.38 g per 100 g fresh muscle and tropomyosin nitrogen content is 1.76% of total nitrogen. According to BAILEY's report[16], tropomyosin content is 0.47 g per 100 g fresh rabbit muscle and 0.21 g fresh pig cardiac muscle.

3. Ultracentrifugation

The solutions are examined by the ultracentrifuge (Spinco, Model E) at 60000 r.p.m./min. The tropomyosin is dissolved in a phosphate buffer of ionic strength 0.1 containing 0.25 M-sodium chloride. The pH of the solution is 7.1. The values of the sedimentation constants are corrected to 2.0°C and to a water basis.

The preparation of tropomyosin appears homogeneous as a result of ultracentrifugation. It sediments as a single fairly symmetrical peak whatever the concentration (Fig. 3). The values of the sedimentation constants at different concentrations are shown in Fig. 4 and Table 3; the sedimentation constant extrapolated to zero concentration is $3.20 \times 10.^{-13}$

When the sedimentation constant of squid tropomyosin is compared with that of crap and rabbit tropomyosins, it is seen that this tropomyosin sediments faster and shows higher sedimentation constants than the others.



Fig. 3. Sedimentation diagram of squid tropomyosin

A. 0.75% protein concentration B. 0.50% protein concentration C. 0.37% protein concentration D. 0.33% protein concentration E. 0.25% protein concentration
a. 10 min. after full speed, **b, c, d,** and **e** are after time intervals of 8 min. (Migration to left)

Table 3. Sedimentation data for tropomyosin at different concentrations in solution (I=0.35. pH 7.1)

Squid		Carp*		Rabbit**	
Protein conc. (g/100ml)	S_{20} corr. $\times 10^{-13}$	Protein conc. (g/100ml)	S_{20} corr. $\times 10^{-13}$	Protein conc. (g/100ml)	S_{20} corr. $\times 10^{-13}$
0.25	3.16	0.22	2.59		
0.33	2.97	0.34	2.73		
0.37	2.77	0.44	2.52	0.60	2.60
0.50	2.81	0.58	2.57	0.653	2.55
0.75	2.36	0.89	2.22	0.665	2.51
Extrapolate	3.20	Extrapolate	2.85		

* HAMOIR[21] ** BAILEY[16]

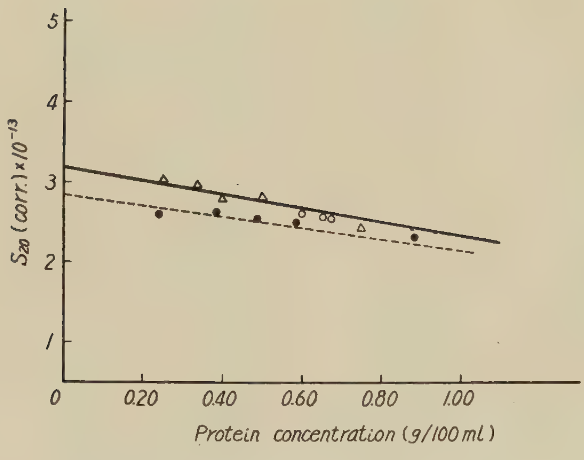


Fig. 4. Variation of S_{20} (corr.) for tropomyosin

Upper curve: squid tropomyosin Lower curve: rabbit and carp tropomyosin △ squid, ○ carp, ● rabbit, carp and rabbit values taken from HAMOIR[21] and BAILEY[16]

4. Electrophoresis

The electrophoretic behavior of protein is greatly effected of the kind of buffer, pH, ionic strength and electric field strength. It is necessary at least that results obtained over a range of pH values and varied buffer ionic strengths be adopted as the criterion of protein purity.[24]

The experiments are performed with TISELIUS apparatus (Hitachi Seisakujo, cell size; 2×15×50 mm). They should be continued about 2–3 hrs. at 4°C and potential gradient is 3.23 volt/cm. pH is determined by glass electrode, Beckman Model-G. Preparations are homogeneous electrophoretically, showing a single component with no impurities (Fig. 5).

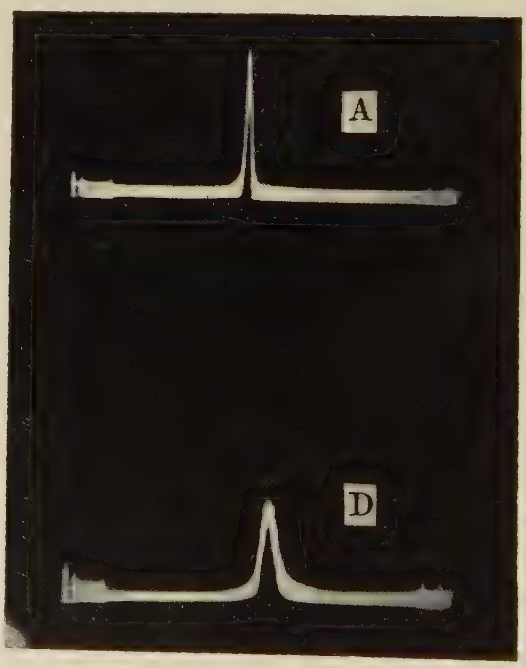


Fig. 5. Electrophoretic diagram of tropomyosin

1% protein concentration, 8640 sec., pH 7.4, I=0.15 (0.1 for phosphate buffer and 0.05 for NaCl), 3.25 volt/cm. A. ascending migration to left, D. descending migration to right

The mobilities found are given in Table 4.

Table 4. Electrophoretic mobilities of tropomyosin (protein concentration 1.0%)

Conditions of electrophoresis		Mobility (10^{-5} cm²/volt/sec)			
		I = 0.15		I = 0.35	
pH		As.	Ds.	As.	Ds.
3.6	Acetate-buffer-NaCl I=0.15(I=0.1 for acetate buffer and 0.05 for NaCl) I=0.35(I=0.1 for acetate buffer and 0.25 for NaCl)	+6.29	+5.82	+4.22	+3.81
3.8		+4.94	+4.48	+3.34	+3.01
4.0		+4.29	+3.90	+2.43	+2.91
4.2		+3.62	+3.29	+1.08	+1.52
6.4	Phosphate-buffer-NaCl I=0.15 (I=0.1 for phosphate buffer and 0.05 for NaCl) I=0.35 (I=0.1 for phosphate buffer and 0.25 for NaCl)	−0.35	−2.73	−2.22	−1.99
7.0		−5.02	−4.50	−3.13	−2.80
7.4		−6.37	−5.71	−4.10	−3.65
7.9		−8.01	−7.91	−5.56	−4.98

The mobility values obtained differ from those of carp and rabbit tropomyosin at I=0.35 and pH 7.1 (Ascending, −4.30, Descending, −3.90 of carp[21]: Ascending, -5.6, Descending, −4.9 of rabbit[25]) and at I =0.15 and pH 7.4 (Ascending -6.90 Descending, −6.20 of carp[21]) and moreover on the acid side I=0.15 and pH 3.5 (Ascending, +6.15, Descending, +5.76 of carp). The mobilities of squid tropomyosin are small in alkaline side and great in acid side. This should be due to the amino acid contents of protein constituent, having basic polar amino acid such as histidine, arginine, lysine and having acidic polar amino acid amide-NH_3 combining with carboxyl radical of acidic polar group such as glutamic acid and aspartic acid.

pH-mobility curve found is given in Fig. 6, but the range from pH 4.2 to 6.4 was not used in the experiment on account of isoelectric zone.

Isoelectric point lies near pH 5.4 in this figure and from the obtained maximum turbidity of solutions by A. K. A.-Type photonepherometer it lies near pH 5.6. Isoelectric point lies somewhat to the alkaline side comparing with rabbit tropomyosin pH 5.1.[16]

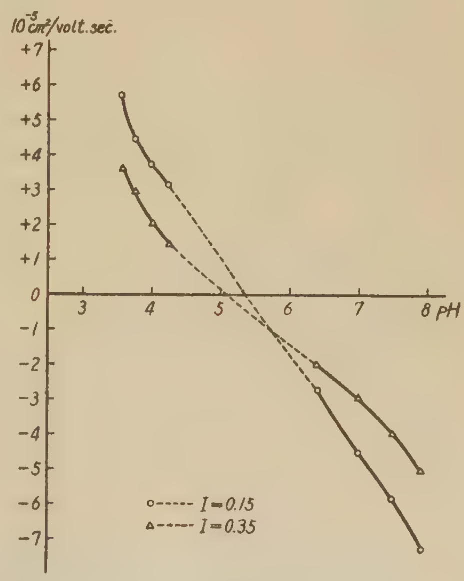


Fig. 6. pH–mobility curve for tropomyosin from descendings

5. Phosphorus and Ultraviolet Absorption

Tropomyosin and nucleotropomyosin have been crystallized from carp muscle by HAMOIR[21] both proteins have the same electrophoretic mobility, however, are different in phosphorus contents and ultraviolet absorption.

The total phosphorus content is determined by the method of CHAIN modified by BINENBLUM.[26]

The ultraviolet absorption spectrum of protein dissolved in the phosphate sodium chloride buffer of pH 7.2 and I=0.35 is measured with a Shimadzu spectrophotometer.

Table 5. Total phosphorus content of squid tropomyosin compared with carp and rabbit tropomyosin

	Squid	Carp*	Rabbit**
Tropomyosin	0.07%	0.20%	0.02%
Nucleotropomyosin	—	0.47%	—

* HAMOIR[21] ** BAILEY[16]

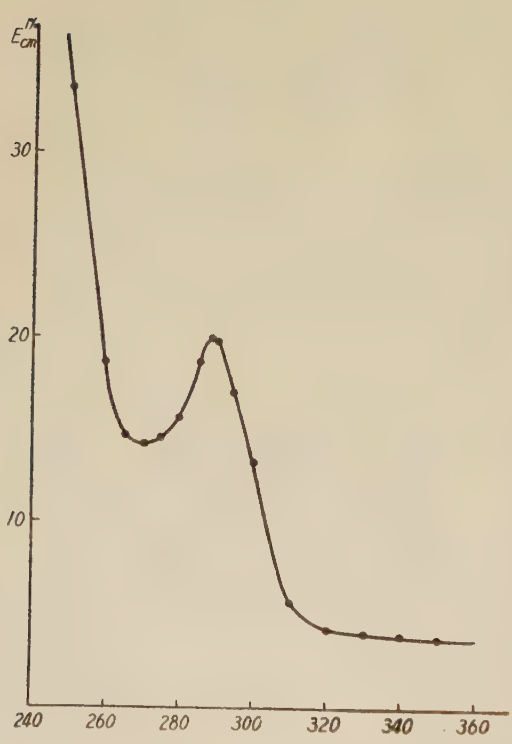


Fig. 7. Ultraviolet absorption spectrum of tropomyosin in a phosphate NaCl buffer of pH 7.1 and I=0.35

The phosphorous content and ultraviolet spectrum found are given in Table 5 and Fig. 7.

The squid tropomyosin has a small total phosphorus content compared with carp tropomyosin and does not show the absorption spectrum of nucleic acid at 260 mμ.

6. Amino Acid Composition

The hydrolysis of the protein is performed in sealed glass tubes evacuated to about 12 mm pressure in an oven at 105°C with 100 volume of 5 N-HCl, to prevent the formation of a large amount of insoluble black humin[28] which is due to the presence of air during hydrolysis. The hydrolysate is filtered by glass filter, and excess HCl is removed by repeated distillation in vacuo. The hydrolysate is made up to 10 ml.

Cystine and cysteine are determined chromatographically as cysteic acid using the perfomic acid oxidation.[29] Sample is

oxidized at 0°C for 4 hours and perfomic acid is removed in vacuo at 30–40°C. The residue is hydrolyzed by 6 N-HCl for 20 hrs. Almost all of HCl is removed by distillation in vacuo and finally neutralized by N-NaOH.

Micro-Kjeldhal determination on the hydrolysates gives the nitrogen value on which percentile values are based. To convert to terms of protein weight, the nitrogen value of 16.68% is used.

The chromatography of the hydrolysate is performed on column of Dowex-50 and Dowex-2. A 0.9×100 cm column of Dowex-50 resin is used for the acidic and neutral amino acid and a 0.9×15 cm of the same resin for the basic amino acid. The 0.5 ml hydrolysate as 5 mg original protein is employed. On the determination of tyrosine and phenylalanine, the fraction of tyrosine plus phenylalanine is obtained by 0.9× 15 cm column chromatography owing to the difficulty of regulating temperature.

Tyrosine is analyzed by the ultraviolet spectrometer, and phenylalanine is determined by subtraction of the tyrosine value from the tyrosine plus phenylalanine value obtained by chromatography.

A 0.9×15 cm column of Dowex-2 resin is used for the cysteic acid and the 1 ml hydrolysate as 5 mg original protein is worked by 0.1 N chloracetic acid for effluent buffer.

Fractions (1 ml) are collected using a Mitamura automatic fraction collector equipped with a drop-counting device. The samples are analyzed by the photometric ninhydrin procedure of MOORE & STEIN.[30] Reading of optical density is made with a Shimadzu spectrophotometer at 570 mμ (proline at 440 mμ). The color intensities are corrected for asparic acid, serine, alanine, valine, tyrosine and phenylalanine according to MOORE & STEIN,[27] for glutamic acid, methionine, glycine, lysine, histidine, ammonia, arginine and cysteic acid according to SCHRAM, MOORE

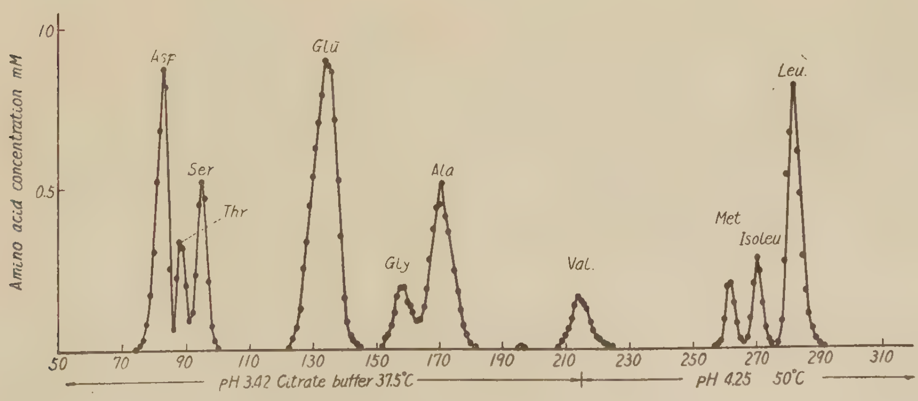


Fig. 8. Tropomyosin Dowex–50 0.9×100 cm

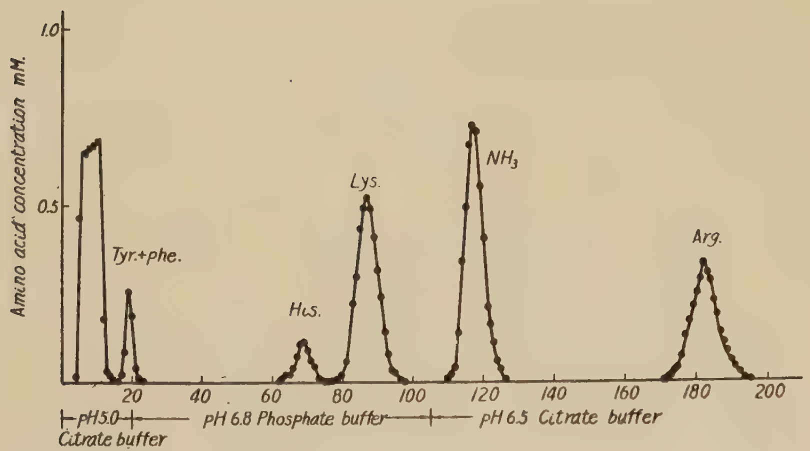


Fig. 9. Tropomyosin, Dowex-50 0.9×15 cm

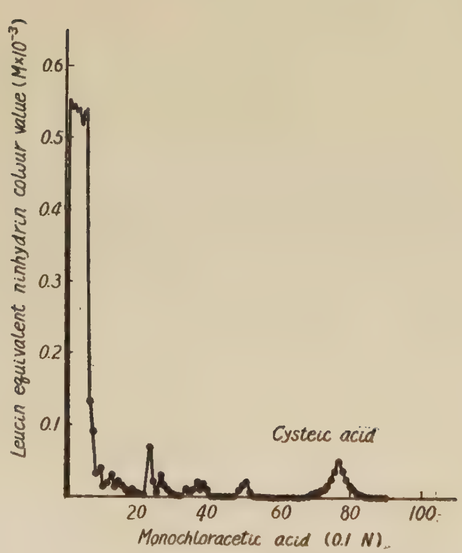


Fig. 10. Tropomyosin Dowex-2 0.9×15 cm

& BIGWOOD,[29] and MOORE & STEIN.[30] The obtained results are shown in Figs. 8, 9, and 10.

The separation of aspartic acid, threonine and serine is imperfect because of no addition of detergent to buffer. The variation on the base line of effluent curve was caused by the incompleteness of the hydrolysis ; small artifact peaks are recognized before the valine.

Compounds in which the carboxyl group of isoleucine or valine is coupled in peptide linkage are not easily hydrolyzed by HCl,[31][32][33] these peptides are effluted before the valine peak.[28] The small amount of valine and isoleucine in Table 6 should be due to this effect.

Certain amino acids, such as aspartic acid, threonine, serine and lysine are labile to acid hydrolysis and especially threonine and serine, and the amounts of ammonia increase with hydrolysis arise from these amino acids.

The corrections are applied to aspartic acid (+5%), threonine (+7%), serine (+10%) and lysine (+8%), moreover to ammonia (−18%). The recovery of glutamic

acid and cysteic acid by chromatography analysis are respectively about 97% and 90%,[13] and corrections are applied to these amino acids. Obtained result is shown in Table 6.

Table 6. Amino acid composition of squid tropomyosin

	Amino acid N %	Amino acid weight %	Amino acid residue %	Amino acid mol number into 10^5 g protein	
				Squid	Rabbit**
Cystine/2	0.46	0.66	0.56	5.5	6.5
Aspartic acid	7.69	12.20	10.54	92	89
Threonine	2.54	3.61	3.07	30	28
Serine	4.28	5.35	4.43	51	40
Glutamic acid	16.42	28.80	25.28	196	211
Proline	0	0	0	0	0
Glycine	2.20	1.97	1.50	26	12.5
Alanine	7.36	7.82	6.24	88	110
Valine	1.92	2.69	2.28	23	38
Methionine	1.34	2.38	2.09	16	16
Isoleucine	1.52	2.38	2.06	18	29
Leucine	7.32	11.49	9.90	88	95
Tyrosine	0.62	1.34	1.21	7	15
Phenylalanine	0.60	1.18	1.05	7	3.5
Histidine	2.89	1.78	1.56	11.5	5.5
Lysine	13.09	12.13	10.63	83	110
Arginine	21.19	10.99	9.85	63	42
Tryptophane	0	0	0	0	0
Amide-NH_3	7.31	1.48*	1.39*	(87)	(64)
Total	99.56	106.77	92.25	805	851

* Omit. from the total value ** KOMINZ *et al.*[18]

As shown in Table 6 in spite of good recovery of amino acid nitrogen, 99.56%, the recovery of amino acid residue is only 92.25 per cent and these results should be caused by the fact that the oxyamino acid is decomposed to ammonia.

Tropomyosin, which contains a large amount of acidic and basic polar amino acid residues and no tryptophane and proline, is an unique protein.

7. The Adenosine Triphosphatase (ATP-ase) Activity of Tropomyosin

Since ENGELHARDT & LJUBIMOWA[11] indicated the mutual relationship between adenosine triphosphate (ATP), which possesses a large amount of chemical energy, and myosin, which is a contractile material, the studies in this field have been active.

Myosin and actomyosin have the ATP-ase activity which splits off the terminal phosphate of ATP. The chemical energy which produced by this reaction is used for contraction of myosin or actomyosin.

There are divergences of opinion as to whether myosin is identical with ATP-ase, or whether the ATP-sae activity is combined tightly with myosin or actomyosin. The ATP-ase activity of myosin depends on its charge which in turn depends on the ions. Though in the absence of salts, myosin is inactive as ATP-ase, it is inactive also if there is too large a quantity of them.

Ca-ion strongly promotes and Mg-ion strongly inhibits the ATP-ase activity of myosin, while both Ca-ion and Mg-ion promote the same enzymic activity of acto-myosin. For the existence of Ca-ion there are two optimum pH of ATP-ase ; they are pH 6.5 and 9.2. On the other hand, it is suggested that the tropomyosin is a prototype of the much larger myosin molecule and may conceivably be a unit utilized in the elaboration of myosin itself.

An attempt was made to ascertain whether the crystalline tropomyosin has ATP-ase activity or not, under the addition of Ca- or Mg-ions to reaction medium as the activator of ATP-ase. Range of hydrogen ion concentration of experiment is chosen pH 6.5–pH 9.5 and as the buffer solution borate-boric acid buffer are chosen. The concentration of adenosine triphosphate solution is 978γ/1 ml.

As the tropomyosin solution 0.1% crystalline squid tropomyosin solution in 0.1% KCl (pH 7.2) is prepared. Following instructions are to be observed : the reaction temperature is 30°C, after 5 min add trichloracetic acid solution and stop enzymic action.

The free phosphoric acid of this solution is estimated by the FISKE-SUBARROW method.[37]

The obtained results are shown in Table 7.

Table 7. ATP-ase activity of squid tropomyosin

pH	Mg-ion		Ca-ion	
	Optical density measured	Optical density corrected	Optical density measured	Optical density corrected
6.71	0.170	−0.025	0.195	−0.010
7.61	0.179	−0.016	0.220	+0.010
7.93	0.192	−0.003	0.225	+0.020
8.27	0.190	−0.005	0.220	+0.015
8.67	0.180	−0.015	0.239	+0.024
8.87	0.210	+0 025	0.224	+0.019
9.07	0.170	−0.025	0.205	+0.000
9.23	0.169	−0.026	0.200	−0.005

From the above results the author is able to recognize the following facts :

Mg-ion as the activator of the ATP-ase activity of squid tropomyosin shows negative effect, while Ca-ion as the activator shows positive effects but within the range of experimental error.

From the above results it is deduced that the squid tropomyosin has no ATP-ase activity.

III. DISCUSSION

In the previous chapter the results of the present studies on the squid tropomyosin are described. In comparing the physicochemical and chemical properties of squid

tropomyosin with those of rabbit tropomyosin, which have been already reported by BAILEY, there are many differences between these two tropomyosins. To have resistance against denaturation, and to separate in crystalline form are common properties. However, in detail, there are also differences in many points, for example, in the process of crystallization and the form of crystal. The crystalline form of rabbit tropomyosin which was isolated by BAILEY[16] is a quadrilateral plate or hexagonal plate, but the squid tropomyosin is a needle. The sedimentation velocity of tropomyosin was measured by ultracentrifugal method, and from this value sedimentation constant is calculated. The squid tropomyosin has a higher value of sedimentation constant than the rabbit. This means that the apparent molecular weight of the squid tropomyosin is higher than the rabbit.

The electrophoretic mobility of the squid tropomyosin is small on the alkaline side and great on the acid side, and the isoelectric point lies somewhat to the alkaline side as compared with rabbit tropomyosin. These facts mean that the amino acids which compose tropomyosin are different in squid from those in rabbit muscle.

The amino acid composition of squid and rabbit tropomyosins are shown in Table 8: both tropomyosins do not have tryptophane and proline, and the other amino acids contents are obviously different between them.

Table 8. Comparative amino acid value of tropomyosin from squid and rabbit muscle

	Amino acid N%		Amino acid weight %		Amino acid mol numbers into 10^5g protein	
	Squid	Rabbit*	Squid	Rabbit*	Squid	Rabbit*
Cystine/2	0.46	0.55	0.66	0.78	5.5	6.5
Aspartic acid	7.69	7.46	12.20	11.84	92	89
Threonine	2.54	2.35	3.61	3.33	30	28
Serine	4.28	3.36	5.35	4.20	51	40
Glutamic acid	16.42	17.70	28.80	31.00	196	211
Proline	0	0	0	0	0	0
Glycine	2.20	1.05	1.97	0.94	26	12.5
Alanine	7.36	9.20	7.82	9.78	88	110
Valine	1.92	3.18	2.69	4.48	23	38
Methionine	1.34	1.34	2.38	2.38	16	16
Isoleucine	1.52	2.43	2.38	3.80	18	29
Leucine	7.32	7.46	11.49	12.44	88	95
Tyrosine	0.62	1.26	1.34	2.72	7	15
Phenylalanine	0.60	0.29	1.18	0.58	7	3.5
Histidine	2.89	1.38	1.78	0.85	11.5	5.5
Lysine	13.19	18.40	12.13	16.06	83	110
Arginine	21.19	14.10	10.99	7.30	63	42
Tryptophane	0	0	0	0	0	0
Amide-NH_3	7.31	5.35	1.48	—	(87)	(64)
Total	95.56	96.86	106.77	112.45	805	851

* KOMINZ *et al.*[18]

About the non-polar amino acid in the squid tropomyosin there is a large content of serine, glycine and phenylalanine and less content of isoleucine and tyrosine in comparison with rabbit. Regarding the amino acids which form the acidic group in

the squid tropomyosin, there is much aspartic acid and less glutamic acid comparing with rabbit, but the differences of the respective amino acids contents are not very great.

Regarding to the amino acids which form the basic group in the squid tropomyosin, there is much content of arginine and histidine and less of lysine in comparison with rabbit.

As the amount of any one amino acid has so little significance for the interpretation of both tropomyosins and of biogenetic relationships, it is most profitable to compare whole groups of amino acids expressed as percentage of the total residues as recorded in Table 9.

Table 9. Tropomyosin amino group percent of total residue

	Squid		Rabbit*	
Free-acid group	25.0	44.6	27.8	46.3
Base group	19.6		18.5	
Hydroxy group	10.9		9.8	
Amide group	10.8		7.4	
Polar group	66.9		64.3	
Non-polar group	33.1		35.7	

* Bailey[16]

In both tropomyosins, the amino acids with non-polar side chain comprise 33% (squid) and 35% (rabbit) of the total; the polar side chains are calculated as 67% (squid) and 64% (rabbit). In terms of charges of both negative and positive type, the values of 44.6% (squid) and 46.3% (rabbit) for tropomyosins are higher than any other protein (myosin 34%, fibrinogen 26%, edestin 27% lactoglobulin 28%, casein 23%, insulin 23%, ovalbumin 20% and zein 3%).

Comparing after the subtraction of free acidic and basic amino group in both tropomyosins, squid tropomyosin is calculated as 5.4% and rabbit 9.3%. According to these values, the differences of isoelectric point and electrophoretic mobility seem to be obvious.

From the result of examination of amino acid content of squid tropomyosin, if one molecule of tropomyosin contains 3 molecular residues of half cystine which is present in the smallest amounts, the amounts of amino acid residues and calculated molecular weight are as shown in Table 10 and average molecular weight is 54300±200.

The molecular weight of rabbit tropomyosin obtained from osmotic pressure by Tsao *et al.*[34] is 53000.

The molecular weight of rabbit tropomyosin calculated from the result of amino acid content is 53000 according to Kominz *et al.*[18]

Thus squid tropomyosin shows different behavior in respect to many properties such as crystalline forms, sedimentation constant, electrophoretic mobility, isoelectric point, phosphorus and amino acid composition when comparisons are made with rabbit and carp tropomyosin.

Table 10. Molecular weight and molecular number of tropomyosin

	Amino acid residue	Minimum molecular weight	Number of residue	Molecular weight
Cystine/2	0.56	18204	3	54600
Aspartic acid	10.54	1091	50	54500
Threonine	3.07	3300	16	52800
Serine	4.43	1964	28	55000
Glutamic acid	25.28	551	107	54700
Proline	0	0	0	0
Glysine	1.50	3811	14	53400
Alanine	6.24	1139	48	54700
Valine	2.28	4355	12	52300
Methionine	2.09	6269	9	56400
Isoleucine	2.06	5511	10	55100
Leucine	9.90	1142	48	54800
Tyrosine	1.21	13521	4	54100
Phenylalanine	1.05	13999	4	56000
Histidine	1.56	8719	6	52300
Lysine	10.63	1205	45	54200
Arginine	9.85	1585	34	53900
Tryptophane	0	0	0	0
Amide-NH_3	1.39	1151	47	54100
Mean				54300±200

V. CONCLUSION

Tropomyosin which has more stability to denaturation is newly crystallized from squid muscle. It appears homogeneous upon electrophoresis and ultracentrifugation. Comparing with BAILEY's rabbit tropomyosin,[16] there is no difference in nitrogen content (squid 16.68, rabbit 16.70%), but there is difference in phosphorus content, (squid 0.07 %, rabbit 0.02 %).

As the squid tropomyosin is low in amount of total phosphorus content and does not show ultraviolet absorption of nucleotropomyosin at 260 mμ, it is concluded that it is not a nucleotropomyosin.

Squid tropomyosin shows higher sedimentation constant than rabbit and carp tropomyosins ; the sedimentation constant extrapolated to zero concentration is 3.20×10^{-13} (rabbit and carp, 2.85×10^{-13}).[21]

The electrophoretic mobility of squid tropomyosin is smaller on the alkaline side and greater on the acid side than corresponding qualities of rabbit and carp tropomyosins ; the pH of isoelectric point of squid tropomyosin is 5.6 (rabbit, 5.1).[16]

In amino acid composition, there is difference in the content of each amino acid, especially in acidic amino acid and basic amino acid : squid tropomyosin is the more rich in content of aspartic acid, arginine and histidine and is less rich in glutamic acid and lysine than the rabbit tropomyosin.

The average molecular weight of squid tropomyosin calculated on the bases of amino acid composition, is 54300±200, and the molecular weight of rabbit tropomyosin obtained from osmotic pressure and from amino acid composition, both, is 53000.[34]

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昭和 30 年 12 月 15 日　印　刷
昭和 30 年 12 月 31 日　発　行

北海道大學水産学部
函館市港町 253 番地

印刷所　笹気出版印刷株式会社
仙台市堤通 27 番地

MEMOIRS
OF THE
FACULTY OF FISHERIES
HOKKAIDO UNIVERSITY